# COMPOUND IDENTIFICATION OF THE ESSENTIAL OILS OF *LIGUSTICUM GRAYI* ROOTS FROM GOLD LAKE BY GC-FID/GC-MS AND GENERAL COMPARISON TO *LIGUSTICUM PORTERI*

A Thesis

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by

Cresterlynn Apelin Cordero

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Approved by:

_____, Committee Chair
Dr. Mary McCarthy Hintz

_____, Second Reader
Dr. Roy Dixon

_____, Third Reader
Dr. Tom Savage

_____
Date

Student: Cresterlynn Apelin Cordero

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Department of Chemistry

Abstract

of

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*Ligusticum grayi* (syn: *Ligusticum cusickii*), also commonly known as oshála or Gray's lovage, is used among Native California tribes, such as the Atsugewi tribe, as a relief for upper respiratory congestion, analgesic, gastrointestinal and pancreas problems. The purpose of this study is to identify the chemical constituents in the essential oils of *L. grayi*. Identification is important because so many of the ligusticum species are used interchangeably in medicine. Since *L. grayi* has not yet been characterized chemically, *L. porteri* was used as a standard and compared in order to assure accuracy and proper technique. To determine the best extraction method, identical extraction and analytical procedures were performed on both *L. grayi* and *L. porteri* using several different extraction methods. These solvent extraction methods included using hexane, ethanol, dichloromethane, soxhlet extraction with hexane, and solvent assisted flavor extraction (SAFE). Chemical identification was achieved using gas chromatography with flame ionization detection (GC-FID) and gas chromatography with mass spectrometry (GC-MS), comparing retention indices and mass spectra to those of known compounds.

The SAFE method produced the best extraction method, resulting in identification of 31 compounds for *L. grayi* (154 total peaks) and 46 compounds for *L. porteri* (160 total peaks) than soxhlet (9 identified of 53 peaks for *L. grayi* and 7 identified of 49 peaks for *L. porteri*) or hexane (4 identified of 8 total peaks for *L. porteri*) extraction. Chemical resemblance between *L. grayi* and *L. porteri* was 60%, consisting mainly of monoterpenes, sesquiterpenes, thymol and carvacol ether.

_____, Committee Chair
Dr. Mary McCarthy Hintz

_____
Date

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# Chapter 1

# INTRODUCTION

## 1.1 Introduction and Scope of Study

### 1.1.1 *Ligusticum grayi*

*Ligusticum grayi* (syn: *Ligusticum cusickii*) is also commonly known as oshála (Brounstein, 1993), Wild Parsley (Mead, 1972), Wild Plum (Mead, 1972) Gray's lovage (Hrusa, 2001), Gray's wild lovage (Hrusa 2001), sheep wild lovage (Johnson, 1999), Gray's licorice root (PLANTS 2001) and kishwoof (Foster & Hobbs, 2002). Ligusticum comes from "Liguria", an area in Italy where lovage abounds and a related genus, Levisticum, also called lovage, was first located (Britton & Brown, 1897, Blackwell, 2006). *L. grayi* is named after an American botanist, Asa Gray (1810-1888), who wrote the 1848 Gray's Manual of Botany and was also a professor at Harvard University (Fagan, 2006). Licorice root is a common name for *L.grayi* because of the odor of bruised herbage (Ross & Chambers, 1988).

*L. grayi* is in the order Umbellales, family Umbelliferae (Apiaceae) that consists of 300 genera and 3,000 species worldwide, and is one of twenty five species in the genus Ligusticum (Hickman, 1993). About twenty of these species are native to the Northern Hemisphere (Britton & Brown, 1897). The Umbelliferae family derives from the new Latin word *umbella*, meaning, "shade" and defined as "plants bearing umbrellas" (Lawton, 2007). Recently, Umbelliferae was renamed Apiaceae; the genus name derives from the classical Greek word for celery (Lawton, 2007). Members of the Ligusticum

genus as part of the Apiaceae or Umbelliferae (celery, carrot and parsley) family, are glabrous (hairless) perennials that are usually branched with hollow stems, aromatic roots, ternately compound leaves, and large compound umbels of white flowers (Britton & Brown, 1897). Therefore, *L. grayi* will be mentioned as either being part of the celery, carrot or parsley family, depending on the reference.

Gray's lovage, *L. grayi*, can be found in eastern Washington, Nevada, Montana, Oregon, western Idaho, and northeastern California as shown in **Figure 1** (Moore, 1994). Bioregional distribution in California (**Figure 2**) includes: Klamath Ranges, High Cascade Range, High Sierra Nevada, and Modoc Plateau (Hickman, 1993). Communities of Gray's lovage are found in yellow pine forests, red fir forests, lodgepole forests, subalpine forests, and wetland-riparian tracts (Califlora, 2008). The habitat consists of meadows and slopes and may occur in wetlands or non-wetlands or along riverbanks. The elevation is between 4000-10500 feet (Califora, 2004). Gray's lovage can be found growing large and healthy in both the sub-alpine system as well as the mid-elevation system in open areas. *L. grayi* blooms from July to September and dies off before winter, by November.

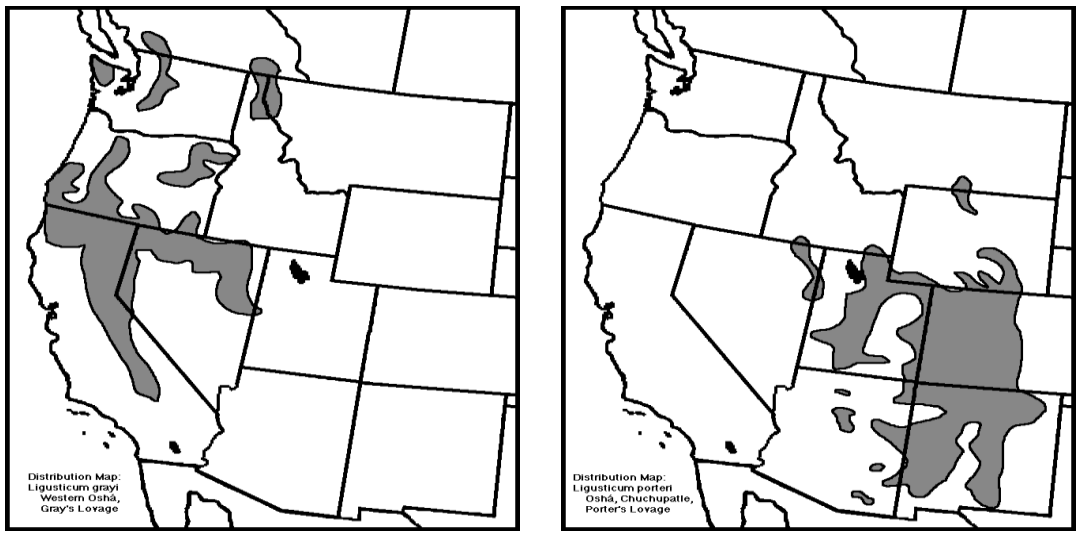


**Figure 1**: Distribution map of a) *L. porteri* b) *L. grayi* in the United States (Moore, 1994)

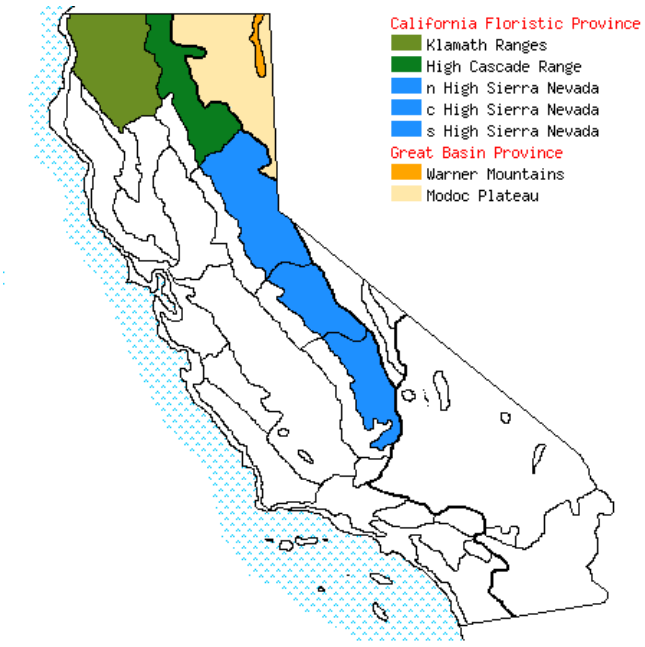


**Figure 2**: Bioregional distribution of Gray's lovage, (Hickman, 1993)

Gray's lovage as shown in **Figure 3** (Moore, 1994), is a dicot and perennial herb with aromatic taproots; 2-6 dm tall with no spots on the stem. The leaves are mostly

basal, dissected, compound, and either ternate (occurring in grooves of three) or ternate-pinnate (feather-like, with rows of leaves on either side of the central stalk). The leaf of Gray's lovage has a petiole of 0.2–0.3 dm. The blade is 1–2.5 dm, oblong to triangular-ovate, double-pinnate or ternate-pinnate; leaflets are 1–4 cm, oblong to ovate, with segments generally narrow and deeply pinnately lobed. *L. grayi* shows typical characteristics of the Umbelliferae family, such as flowers in umbels, inferior ovaries, of two carpels, hollow stems, and leaves without stipules. The flowers tend to be white to pink in compound umbels and the fruits are laterally flattened, oblong to ovate, glabrous, and prominently ribbed to slightly winged with stylopodi (Howie, 1993). The fruit of Gray's lovage is on average 4–5 mm, oblong-ovate with narrowly winged ribs and a concave seed face (Hickman, 1993).

The roots of Gray's lovage range from 15-50+ cm long and range from 5-25 cm wide. Two to three thinner medium roots may branch from the main root with multiple branching towards the tip of each root. When *L.grayi* roots are crushed or chewed, the interior is a grayish white color that dissipates a strong celery scent. Gray's lovage looks very similar to and is sometimes confused with poison hemlock, *Conium maculatum*, but the strong celery scent makes *L. grayi* distinctive. Additionally, Gray's lovage's characterization and medicinal reputation is based on its aromatic properties. The fruits and especially roots of this family are aromatically stimulating, having diuretic and carminative action (Grieve, 1971).


**Figure 3**: Gray's lovage (*Ligusticum grayi*):(Moore, 1994)

### 1.1.2 History and uses

*L. grayi* was recognized as a distinct species by Coulter and Rose (Coulter and Rose, 1889) after its collection August 20, 1889 by Prof. E.L. Greene on open ground near timberline (5,000 ft altitude), on Mt. Rainer, Washington and by Prof. John Macoun, August 5, 1889 in flower on the mountains north of Griffin Lake, B.C., (altitude 6500 ft) (Coulter and Rose, 1889). However, *L. grayi* was first primarily used by the Atsugewi Indians. The western Atsugewi (Atsuge) inhabited heavily wooded areas between Mount Lassen and the Pit River (Garth, 1945). The tribe was divided into two groups, those who settled on Hat Creek (one mile south of the present town of Cassel, in the vicinity of

Mount Shasta) and the other in Burney Valley (Dixon, 1908). In these early reports, the Atsugewi were described as being environmentally determined and expressing traits similar to neighboring Indians, focusing on industrialism, along with the quest for food, which became the dominant feature of the culture (Garth, 1945).

The main source of food for the Atsugewi was acorns, but other vegetables were used with various seeds and berries together with roots. The Atsugewi ate the tender leaves of oshála in the spring, soaking the leaves, then baking them in an earth oven. After baking, they could be stored or eaten with acorn mush as a meat substitute or stored for later use (Campbell, 1999). The leaves of oshála taste like mild parsley. The seeds make a pleasant spice or a pleasure tea. Like the root, these aromatic seeds can be useful medicinally in the form of a tea or tincture for stomach irritability. The roots were also used for hunting by pulverizing the roots and broadcasting the powder in a shallow pond to poison fish (Moerman, 1998, Garth, 1953). Oshála was an important herbal medication of the Atsugewi and used as follows (Moerman, 1998; Garth, 1953):

Analgesic: Roots were used to alleviate pain.

Cold Remedy: An infusion of roots was taken or roots were chewed for colds.

Cough Medicine: Roots were infused or chewed for coughs.

Gastrointestinal/Pediatric: Children took an infusion of roots or chewed for stomach pain.

Panacea: Ailments were cured using roots that were infused or chewed.

In addition to theses traditional uses, herbalists have use oshá in relieving upper respiratory congestion, colds, coughs, and sore throats (Theherbalist, 2008). General

herb sellers and herbalists suggest taking a few drops of tincture followed by some water, or chewing a small piece of the root to cure sore throats from colds and coughs. The root is a very warming herb that increases circulation and promotes sweating and makes it a diaphoretic, useful in the beginning stages of colds and flus (Brounstein, 1993). The aromatic and bitter qualities of this root make it useful for treating mild indigestion, flatulence, stomach irritability, and colic; exciting perspiration; opening obstructions; and for treating the common cold and flu (Grieve, 1971). Another example of its current use is that oshála, as well as true oshá (*Ligusticum porteri)* is known to possess emmenagogual properties, bringing on menstruation (Brounstein, 1993). Therefore, it is imperative that pregnant women or women who want to become pregnant not take this root. These medicinal uses, together with possible anti-bacterial and anti-viral properties (Appelt, 1985), indicate that this herb is an excellent treatment for general infections similar to *Ligusticum porteri*.

Oshála is not commercially cultivated but wildcrafted and is either sold in the form of a powder or dried root, which ranges from $30-35/lb to about $9/ounce (LoveLeaf Garden, Nature Spirit Herbs).

Oshá is the collective name of at least three species: *Lomatium californicum, Ligusticum porteri,* and *Ligusticum grayi* (Kirkpatrick, 2001). The three species have similar medicinal uses but may differ in compound chemistry. According to previous studies, *L. porteri* is considered to be the most potent of the Ligusticums (Brounstein, 1993). Since the chemical composition of *L. grayi* has not been thoroughly analyzed, *L.*

*porteri* will be used for cross-referencing and comparison of compound similarities and differences.

### 1.1.3 Previous research on related plants

Related species include *Ligusticum wallichii* Franch. (*L. chuaxiong* Hort.). This is an important herb in China that is used to invigorate the flow of blood, treat gynecological problems such as dysmenorrhea, treat colds and flu-like symptoms and alleviate pain in the chest and head (Garth, 1953). Studies have shown that *L. wallichii* improves cerebral blood flow, relieves asthma, and has antianginal, anti-inflammatory, and hypotensive properties (Ko, Yang, & Chen, 1996). The roots of *L. sinense* Oliv. or *L. jeholense* (Nakai et Kitag.) have medicinal purposes similar to *L. grayi*, being prescribed for common colds, headaches, and acute lower-back pain (Moerman, 1998; Garth, 1953). The roots of *L. sinense*, used by the Chinese and spread to the northwestern United States, and of *L. angelica* are used widely as stimulating expectorants (Gieve, 1971).

### 1.2.1 *Ligusticum porteri*

Of all the New World species, *Ligusticum porteri*, or true oshá is the most researched. Other common names for *L. porteri* include mountain lovage, Porter's lovage, Porter's wild lovage, loveroot, wild lovage, licorice root, Porter's licoriceroot, Colorado cough root, bear root, Indian root, Indian parsley, wild parsley, mountain

ginseng, mountain carrot, nipo, Empress Of The Dark Forest, chuchupati, chuchupate, chuchupatle, guariaca, hierba del cochino, raíz del cochino, washía (by the Tarahumara in Mexico), yerba de cochino and southern ligusticum [Plants for a Future, 1999, Walker & Hudson, 1993]. (Chuchupate can also refer to "balsamo" or *Myroxylan balsamum* (L.) Harms. in the family Fabaceae, which is a native tree in South America that is used or similar medicinal purposes (Linares, 1986).)

*L. porteri* is found in the American Great Basin, American Southwest (**Figure 1**) and Mexico (Timbrook, 1987). In Utah, the species has been identified in several counties including Beaver, Cache, Carbon, Duchesne, Emery, Garlfield, Grand, Iron, Juab, Kane, Millard, Piute, Sand Juan, Sanpete, Sevier, Summit, Tooele, Uintah, Utah, Wasatch, Washington, and Wayne (Welsh, 1993).

*Ligusticum porteri* populates the high elevations of the Rocky Mountains (USA) and Sierra Occidentalis (Mexico) (Moore, 1994). Populations grow along streams, on open slopes, and in aspen and mixed conifer/deciduous forests (Cronquist et al., 1997). These plants prefer moist fertile ground and tend to grow in groups.

*L. porteri* is distinguished by its taproot, which has fibrous root hairs, and fibrous leaf bases at top. The leaves divide and arch upwards (an Umbelliferae characteristic) where leaves alternate, pinnately divided, into numerous lobed leaflets with leaf stem bases clasping (**Figure 4)**. The five-petaled white flowers are small and arranged in loose umbels. Oval fruits are narrowly winged and have a distinctive odor due to resins produced by the plant (Foster & Hobbs, 2002). Perennial growth of the plant is 20-40

inches tall. *L. porteri* blooms June to August and is usually collected at this time, up to early fall. It withers by winter.

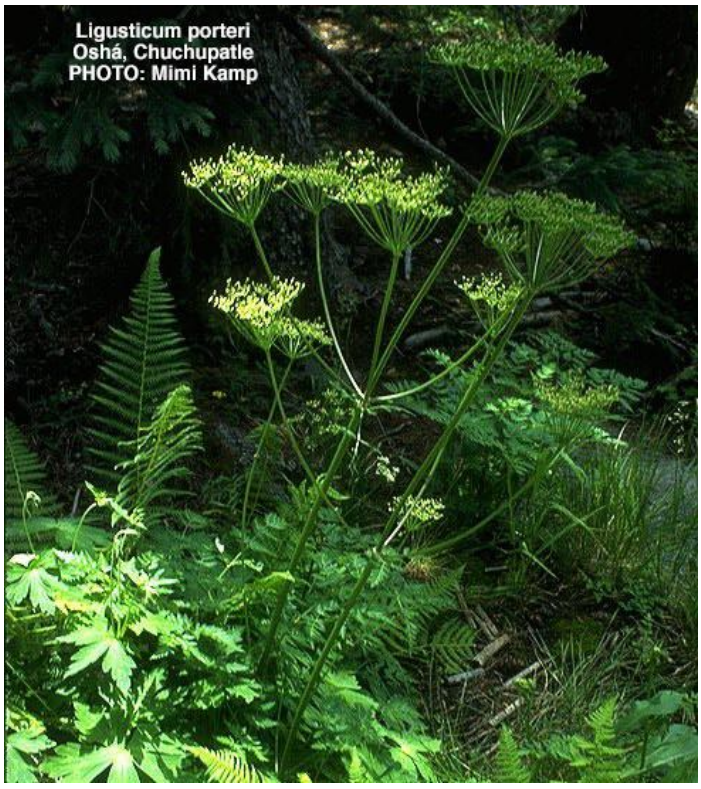


**Figure 4**: Oshá (*Ligusticum Porteri*) (Kamp, 2005)

Within the genus Ligusticum, *L. filicinum*, *L. canbyi*, and *L. tenuifolium* are similar species to *L. porteri* that may be used interchangeably as oshá in the herbal market. Species may be identified according to the structure and number of "rays" in the compound umbel of the flower (Cronquist et al., 1997).

### 1.2.2 History and uses

Oshá root (*L. porteri*) was traditionally used by the Jicarilla Apache in ceremonial smoking blends with tobacco as well as by decoction to soothe sore throats, coughs, headaches and fevers (Linares & Bye, 1987; Opler, 1994). In the Apache Jicarilla language, oshá is called *ha'ich'idéé*. Neighboring White Mountain Apache call it "'Ha 'il chii' gah". It was collected from the Rocky Mountains and advertised for its capability of curing anything. The roots and leaves were scraped to create smaller pieces that could be ingested or used in an infusion (Harrison, 2002).

*L. porteri's* traditional medicinal uses were as a tonic for colds, bronchial infections, fever, poor digestion, sore throats, lung congestion, and spasms. Its current clinical applications are for coughs, bronchial conditions, menstrual pain, placenta retention, fevers, digestive disorders, and toothaches (Native American Botanics, 2004). *L. porteri* is very effective as a tincture of fresh root 1:2, or dry root 1:5, in 70% alcohol. Twenty to sixty drops can be taken sublingually, up to five times per day. As a cold infusion, two to six ounces of fresh root is mixed with water and taken as needed (Moore, 1977).

Oshá is also one of the best treatments for viral infections (Moore, 1997) and is strongly antibiotic, antiparasitical, and antihelminthic (active against intestinal worms) (Moerman, 1998). Oshá means "Bear Medicine". It was called Bear Medicine because the first thing bears do after hibernation is dig their claws into Oshá in order to chew and rub it on their fur to clean the body of any winter parasites (Moerman, 1998). Furanocoumarins such as psoralen and bergapten have been isolated in various

Ligusticum species (Brown, 1977). This is useful because natural furanocoumarins may be effective in vitiliginous skin areas (Soine, 1964). Oshá has been proposed for treating viral infections (Brown, 1977, Appelt, 1985) and may be used as an antitumor agent (Soine, 1964, Appelt 1985). Antifungal and antibacterial activity attributed to the furanocoumarins may be related to the cytotoxic activity (Appelt, 1985).

In general, *L. porteri* can be used as an antiallergen by the use of root tincture or chewed root during histamine flare-ups such as hives; anticatarrhal to help bring up phlegm in the sinuses and throat; antimicrobial for its usefulness on a number of bacteria and viruses; antivenom for bites and stings; antiviral for sore throats from the flu; curative for high-altitude sickness; protective/talisman beliefs for healing powers and protection; and sore throat reliever (Apelt, 1985).

### 1.2.3 Chemical constituents

Phthalides are responsible for *L. porteri's* distinguishable smell and bitter taste, which is similar to celery. Phthalides such as butylidenephthalide (BdPh) and ligustilide have been isolated from several *Ligusticum* species (Gijbels *et al*., 1981). Experiments with BdPh have demonstrated that it can inhibit smooth muscle activity (Ko, 1980). Phthalides are also present in celery seed oil (Uhlig et al, 1987) are reported to have sedative activity (BioActives, 2001), and may be effective cancer chemopreventive agents, with one account describing the reduction of tumor incidences by 67% to 83% in mice (Zheng et al, 1993).

Other chemical constituents found in *L. porteri* include alkaloids, sterols, saponins, lactones such as E- and Z- ligustilides, pinenes, furocoumarins, and sterols (Thopil, 2004). It has been found that the antiviral and antimicrobial component in oshá is (Z)-ligustilide, (**Figure 5);** Linares and Bye, 1987).

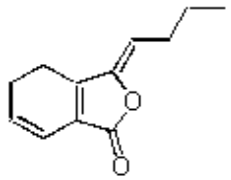


**Figure 5**: Z-ligustilide

**1.3 Purpose**

The goal of this study is to identify the chemical constituents in *L. grayi*. Identification is important because *L. graiyi* has not yet been chemically characterized and so many of the ligusticum species are used interchangeably in medicine. Since *L. porteri* has already been characterized chemically and is from the same genus as *L. grayi*, it was used as a standard to compare, characterize, assure accuracy and implement proper techniques. Identical extraction and analytical procedures were performed on both *L. grayi* and *L. porteri*. Several different extraction methods, including solvent extractions using hexane, ethanol, and ether, soxhlet extraction, and vacuum distillation were assessed. The SAFE-BAENG extraction method was used to further characterize chemicals of higher concentrations of *L. grayi*. Chemical identification was achieved using gas chromatography with flame ionization detection (GC-FID) and gas chromatography with mass spectrometry (GC-MS), comparing retention indices and mass

spectra to those of known compounds. Unidentifiable compounds of high yield will be further analyzed, but are not within the scope of this project.

### 1.4 Examining *L. grayi,* Plant Collection

Ligustum grayi specimens were collected from populations at Bear Creek campsite, Gold Lake, Plumas County, California. Populations were collected from Gold Lake because of L. grayi abundance, ease of collection, close location and the historical use of its land for medical herb collection. Five plants were collected about 0.25 miles away from each other, all around the Bear Creek campsite, as is fully described in the Methods section.

Since oshá is difficult to cultivate, it must be collected from wild stands. To insure the continuance of the stand, the best time for harvesting roots is in the fall, from late August to earlier November. This allows the harvester to reseed the stand. Richard McDonald and Shawn Sigstedt, long time researchers in the field, advise that "We can obtain a 96 percent germination rate from oshá seed. A person can actually regenerate more than the amount they dig, very easily. The trick is to make sure the seeds are ready to fall off the plant. The seeds cannot be green, but need to be brown and dry. Some of the seeds from the umbel probably will have fallen off already. By holding the stem just below the umbel, and then gently breaking off the brittle stalk below with the other hand, a person can usually collect the entire umbel without losing too many seeds. After a soft flat 'step' has been prepared on the hillside (where the seeds will be planted), the individual umbelettes are gently broken off the main umbel. These have five to seven seeds each and are placed upside down on the earthen step, looking much like mini tipis.

The oil duct of the seed needs to be in an upside-down position for optimum germination. Space these out rather closely, since oshá plants like to grow together, intertwining their roots into a large mass. All the planting steps should be carefully covered with light soil and decaying leaves, but not too deeply, perhaps 1/2 to 1 inch is best. Then, as an added precaution, try to protect the newly seeded area with branches, small logs, or whatever is nearby to prevent animals from smashing the new seedlings as they sprout. People should collect oshá only in the fall season, when the seeds are ready to plant." (Philip, 2005)

**1.5 Background of Methodologies**

In order to characterize the compounds in *L. grayi*, it was necessary to use several extraction techniques to determine which yielded the most compounds and the most abundance of each compound. Since the chemical make-up of *L. grayi* was not previously analyzed, methods of purification and identification were developed based on previous research with related plants. Much of the analysis for this thesis was based on testing and developing the best methods specifically for *L. grayi*. One of the aims of this study is to compare the efficacy of several extraction methods, including solvent, soxhlet, and steam distillation. Therefore, a general description of the theory behind these extraction methods is necessary. Description of another particular extraction method, SAFE/BAENG extraction, is also necessary because it is specialized, unique and only used by a handful of scientists. Furthermore, detection methods are also described because of their relevance in identifying the compounds in *L. grayi*.

### 1.5.1 Solvent extractions

Solutes have different solubilities in different solvents, and the process of selectively removing a solute from a solvent mixture is defined as extraction (Shugar, 1996). Extraction of 10 g of root material, with 50 mL of n-hexane, dichlormethane, or ethanol twice, for 2 days each extraction, was the first choice of extraction. Previous research has shown that n-hexane extracts the volatile components better than ethanol does and that ethanol extracts high concentrations of sugar that need to be separated from the nonpolar components (Zschoche *et al*, 1998). The benefit of a simple solvent extraction is the low cost due to accessibility of solvent and apparatus. Compared to advanced extraction techniques, the disadvantage of simple solvent extraction is the low selectivity, low purity, solvent excess, and need for more root plant material to obtain observable peaks via GC-MS analysis.

### 1.5.2 Soxhlet extraction

Another extraction method, soxhlet, can be used to extract solutes from solids using any volatile solvent, which can be either water-miscible or water-immiscible (Shugar, 1996). The solvent is boiled into a specialized condenser, and, when condensed, it drips into a thimble containing the plant material in a reservoir extracting soluble compounds. The solvent fills the reservoir and the adjoining siphon tube until it gets siphoned into the original flask, then goes through another cycle of vaporization and condensation (**Figure 6**). The extraction can continue, unattended, for many cycles.

The benefits of soxhlet extraction include higher yield over simple solvent extraction and ease of extraction methods (Szentmihalyi, 2002). Additionally, since it is a continuous extraction, the experiment can run on its own for many hours. The disadvantages of soxhlet extraction are the low purity and yield and long extraction time (in this case up to 24 hrs). In a recent study comparing soxhlet to new extraction techniques such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE), it was shown that soxhlet extraction is more time-consuming, requires a larger volume of organic solvent, and analyte recovery is less precise (Wang *et al*, 2007).

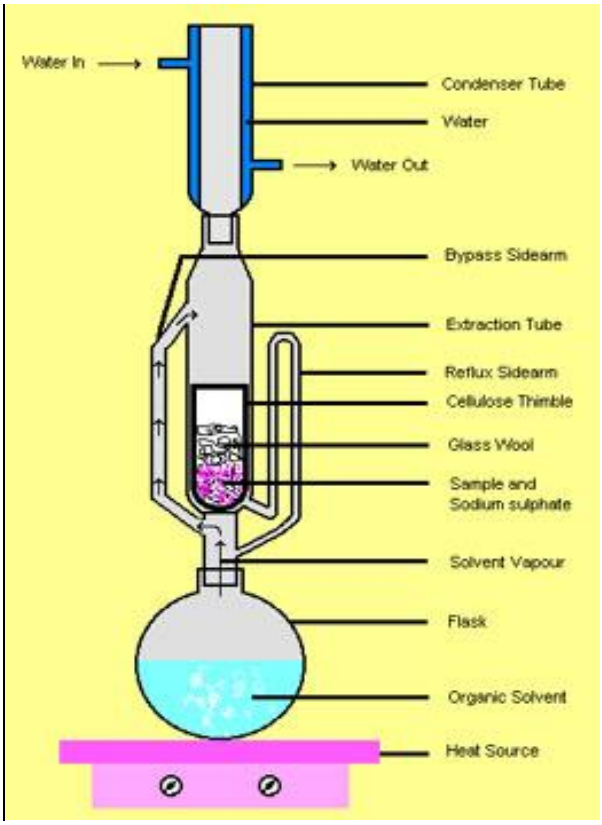


**Figure 6**: Soxhlet extraction apparatus (Bergeron & Benning)

### 1.5.3 Vacuum distillation

The volatile faction, also known as essential oil, is a concentrated hydrophobic liquid that contains the essence or aroma compounds of the plant after extraction. The most widely used method for essential oil extraction is steam distillation. Distillation is a process where the solvent of choice is vaporized, recondensed, and collected in a receiver (the distillate) and the residue (non-vaporized) is left over. However, many plant compounds decompose before they reach high enough temperatures to vaporize. Steam distillation is used to purify and distill essential oils, where organic compounds insoluble in water can be purified at a temperature well below the point at which decomposition occurs. (Shugar, 1996).

The procedure of vacuum distillation starts with plant material in water in a steam distillation apparatus (**Figure 7**). However, a hotplate is used to gently heat the flask in a beaker of water, so that the temperature is not as high as it would be in a steam distillation. The distillate, which includes water and essential oil, is collected in a side-arm flask containing water. The side-arm flask is connected to a vacuum linel the vacuum allows the volatile material to evaporate at a lower temperature. Then the distillate is poured into a separatory funnel and extracted into a nonpolar solvent.

This method has been used since the early 1800's and is the easiest way to collect an essential oil. The advantages include ease of use, low cost of the machinery, and fair purification. There are also disadvantages to steam distillation, such as keeping the right temperature and not overheating the sample. Evaporation times can vary, so it is necessary to monitor the temperature and water levels throughout the experiment.

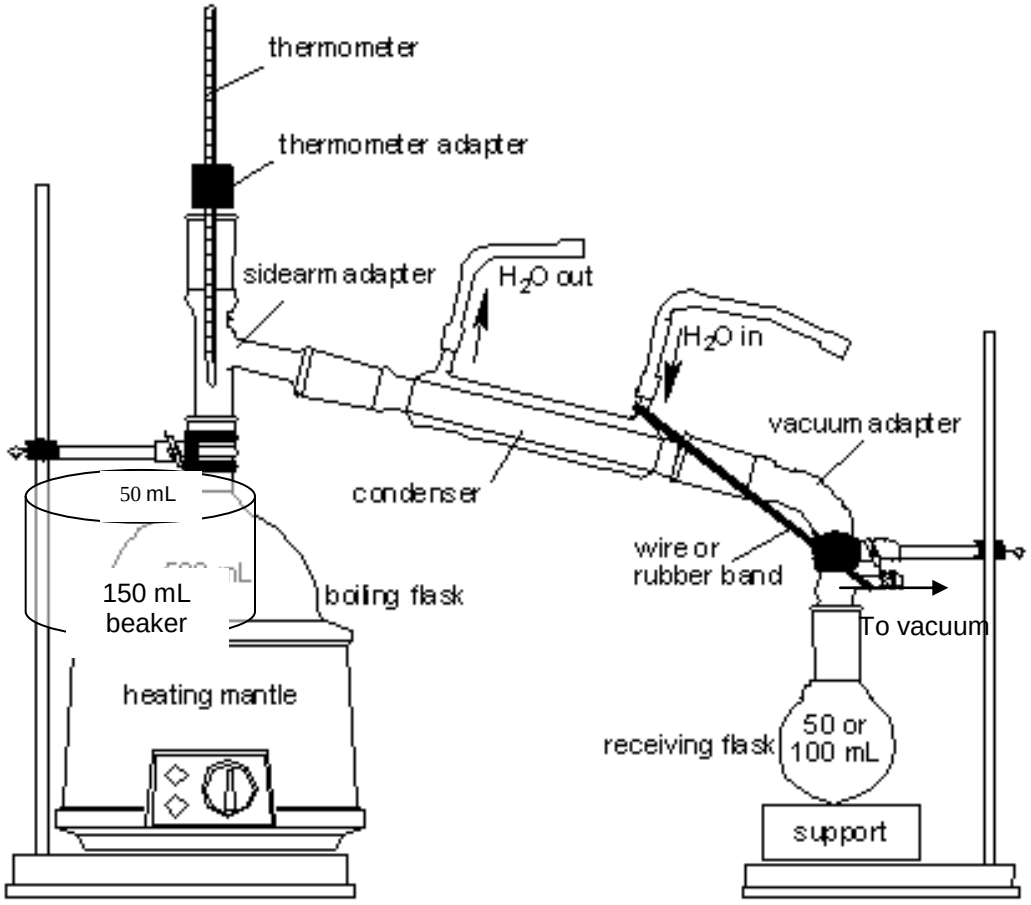


**Figure 7**: Steam distillation apparatus (Burgess, 1981)

### 1.5.4 SAFE/BAENG distillation

Solvent Assisted Flavour Evaporation (SAFE) using the BAENG apparatus (**Figure 8**) acts just like vacuum distillation but with a more volatile solvent and expensive glassware made by Baeng in Germany. SAFE distillation uses an organic solvent such as ether for distillation and liquid nitrogen to condense the distillate. The

distillate and residue have their own compartment during the condensation process and the apparatus is tightly sealed.

The benefit of the SAFE method is the extraction of a wide range of volatiles with higher boiling points. Previous research also state that the SAFE apparatus is the softest methods for preserving added aroma compounds and is a useful method to extract aroma compounds in oily matrices (Engel *et al*., 1999).

Unfortunately, the BAENG apparatus is used only by a handful of people, including the USDA researchers in Emeryville, and the apparatus now costs over $1000. The other disadvantage is the preparation time to clean and set-up the apparatus.

**SAFE-Apparatus used for the isolation of aroma compounds by high vacuum distillation (Solvent Assisted Flavor Evaporation, Engel et al., Eur. Food Res. Technol., 1999, 209: 237-241)**

A dropping funnel
B heatable SAFE-apparatus
C distillation vessel
D water bath (40 C)
E magnetic stirrer
F to high vacuum pump
G safety cooling trap with liquid nitrogen
H vessel for distillate
I cooling trap with liquid nitrogen
J, K laboratory lifting platform

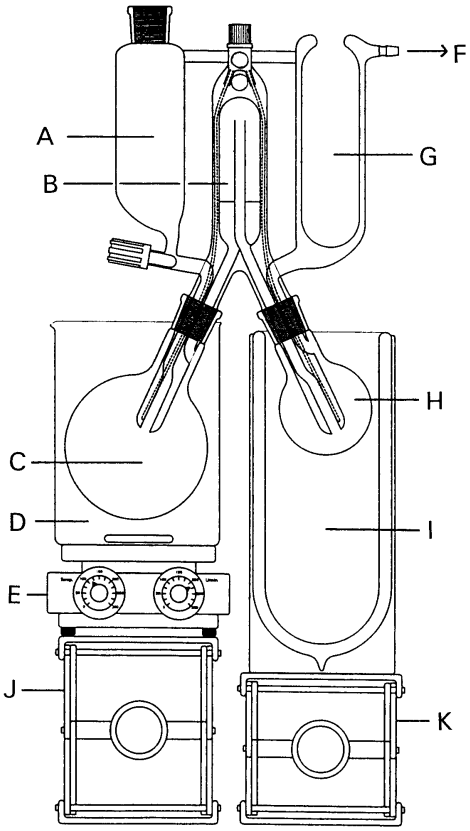


**Figure 8**: SAFE distillation using BAENG apparatus

### 1.5.5 Gas chromatography

Once the essential oil has been extracted from the plant, then separation of the compounds is necessary for identification. Gas chromatography (GC) is a well developed separation method with the sensitivity to detect volatile organic mixtures of low concentrations. Gas chromatography utilizes a column where the stationary phase is made up of miscroscopic layer of polymers or liquid on an inert solid support on the wall of the glass or metal capillary tube, called a column, with a relatively inert gas (such as nitrogen, helium, carbon dioxide or argon) as the mobile phase that moves through the column to a detector, so that a chromatogram is generated that shows the retention times of the molecules (**Figure 9**). The column is housed in an oven so that the temperature can be controlled. The retention time is time taken for the mobile phase to pass through the column. The main reason why different compounds can be separated this way is because of the differential interaction between different compounds and the stationary phase, which determines the rate of migration through the column. Retention time varies with volatility of the analyte, polarity of the stationary phase, column (oven) temperature, carrier gas flow rate, column length, and amount of material injected. Low boiling point compounds travel through the column faster with shorter retention times than more volatile compounds. The main determinant of the volatility of a compound is its size or molecular mass. Also, polar compounds are less volatile than non-polar compounds, so they interact more strongly with a polar stationary phase and hence have a longer retention time than non-polar compounds. High temperatures and high flow rates decrease the retention time and deteriorate the quality of the separation. The higher the

temperature, the more the compound is in the gas phase, interacting less with the stationary phase, decreasing the retention time. If the carrier gas flow is high, the molecules do not have much chance to interact with the stationary phase, so the retention times of all analytes decrease, resulting in poor separation. Furthermore, the longer the column, the better the separation but the greater the retention times. Finally, slow injection of large samples causes poor separation, band broadening and loss of resolution. High temperatures and high flow rates decrease the retention time and deteriorate the quality of the separation. Each of these separation factors must be considered for analysis of each plant sample.

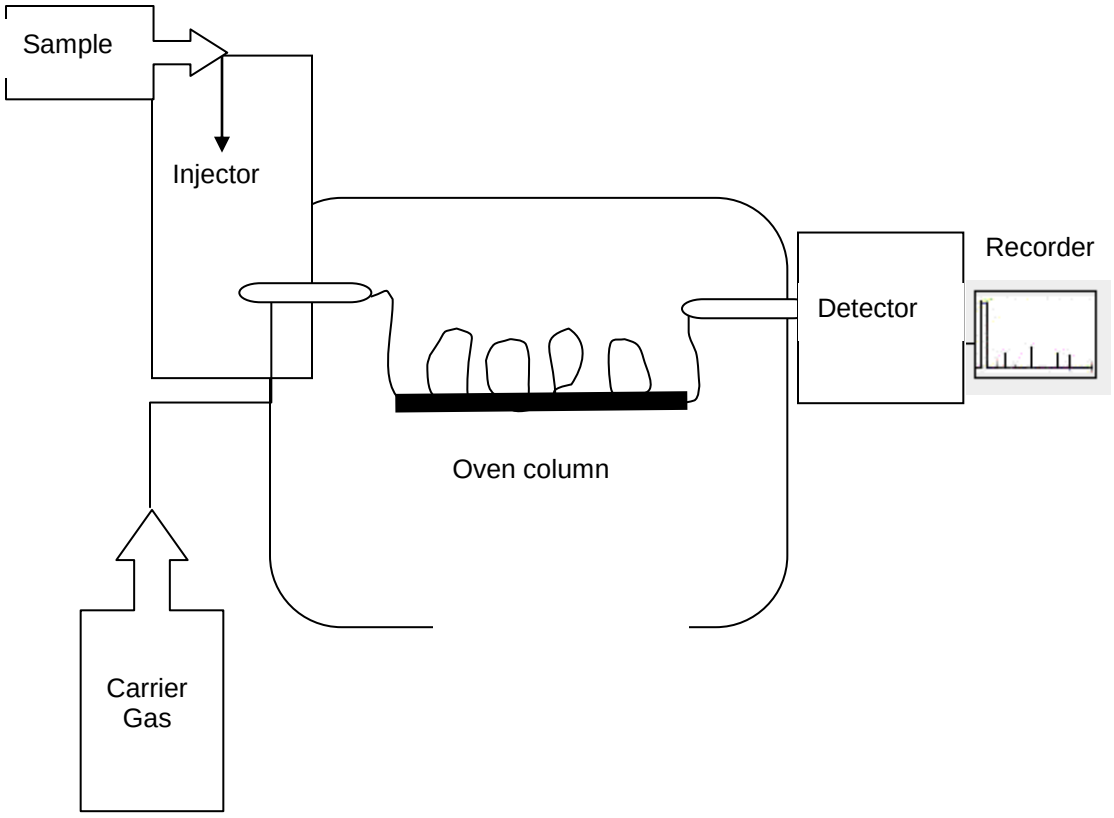


**Figure 9:** Schematic diagram of a gas chromatograph

Among all analytical separation techniques, GC has the most advantages because of its separation efficiency, high-speed analysis, high availability of sensitive detectors, extensive libraries for identification, and ease of use. However, HPLC may be better if many compounds of interest that are thermosensitive or for isolation of a specific compound (Liska, 1996). GC has been the most widely used, for example, in successful isolation of phenylpropanoid derivatives from the essential oil of *Pimpenella aurae.* (Delzar, 2006). Delzar *et al* also state that "... GC-MS is a valuable tool in modern food, medicine, and biological research among aiming at separation and identification of components of organic mixtures, and this method has already been applied successfully for the analysis of terpenoids, especially mono- and sesquiterpenes in various resin oils (Delzar *et al*, 2004). Primary Identification of pthalides and monoterpenes in hexane extracts of *L. porteri* was also accomplished using GC-MS (Delgado *et al*, 1991). Therefore, GC was the method of choice for separation and identification of both *L. grayi* and *L. porteri* constituents because a large range of organic compounds with higher boiling temperatures, such as terpenoids, can be separated and only a small volume of essential oil (1-10 µL) is needed.

### 1.5.6 Detectors: FID and MS

After separation by GC-MS, molecules can be detected in-line by a number of techniques such as the thermal conductivity detector (TCD), thermionic detector (TID), electron capture detector (ECD), atomic emission detector (AED), mass spectrometry detector (MS) or flame ionization detectior (FID). An ideal detector should have

adequate sensitivity, good stability/reproducibility, a linear response to analytes that extends over several orders of magnitude, low cost, short response time, ease of use, similarity of response towards all analytes, and be nondestructive to the sample. MS and FID are the best for this project. Both detectors are powerful tools, the FID best in detecting hydrocarbons, with a detection limit of 1pg/s, and MS tunable to any species, with a detection limit of 0.25-100 pg (Skoog, 2007). Usually, to obtain greater accuracy of compound identification results, researchers use both FID and MS to cross-reference each other (Marriot *et al*, 2000, Shellie *et al*, 2002). This practice is implemented in this thesis project.

FID is best for detecting organic compounds such as proteins or plant samples and is sometimes called the "carbon counter" for its usefulness in detecting molecules with high carbon compositions (like terpenes found in plants). In FID (**Figure 10**), the gaseous eluent from the column is mixed with separately plumbed in hydrogen and air and all are burned on the tip of a jet. After the fuel ($H_2$) and oxidant ($O_2$ in air) are immersed together at the end nozzle head, the flame is lit using an electronic ignitor, The gaseous products enter the detector chamber via the exhaust and are recorded in response to the cations created by combustion in the flame. The charged particles created in the combustion process create a current between the detector's electrodes, which is detected by a sensitive ammeter, then the signal is fed to an amplifier, then to an integrator and finally to a display system. The negative current supply is a high voltage type that supplies the carbon cations with electrons, but the ground is the input of a sensitive current amplifier which counts the electrons passing through it on their way to neutralize

the charge. Indirectly then, for carbon atoms in an H-C molecule, this scheme acts as a "Carbon Counter", as the signal is roughly proportional to the number of carbons oxidized during pyrolysis. Unfortunately, FID is the most sensitive for hydrocarbons but does not respond well to heteroatoms such as oxygen or nitrogen. The signal is lower for carbons that are already partially oxidized. Therefore, MS is used for actual identification of compounds, while FID is used to confirm the number of carbons in the tentatively identified compound.

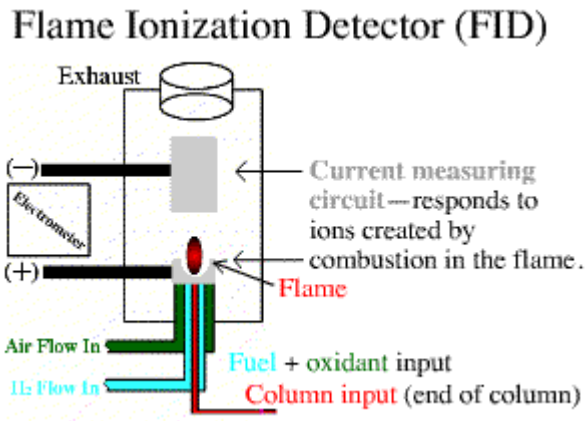


**Figure 10**: Schematic of flame ionization detector (FID), (Chasteen, 2000)

MS detection (**Figure 11**) is used to measure the characteristics of individual molecules. The mass spectrometer converts the molecules into ions so that they can be moved by external electric and magnetic fields. A small sample is ionized to cations by loss of an electron through an ion source (such as an electron-impact source which comes from a heated filament). Then the ions are sorted and separate according to their mass

and charge through a perpendicular magnetic field the magnetic field that deflects the ions into an arc whose radius is inversely proportional to the mass of each ion. Ions of different mass can be focused progressively through an electric current that is proportional to the number of ions arriving, which is amplified and recorded by the detector.

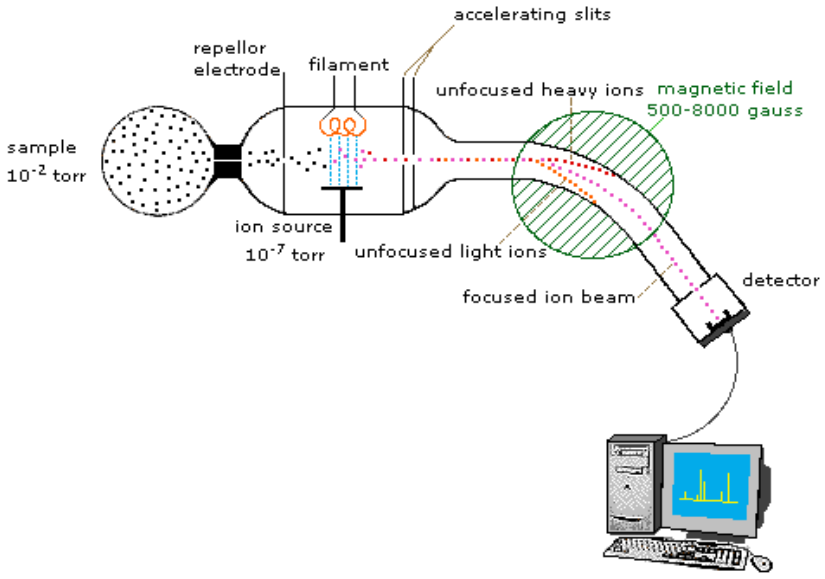


**Figure 11**: Schematic of mass spectrometer (Michigan 2007)

MS is widely used by researchers because it is selective, universal, and sensitive. MS is very useful for identifying compounds and is ideal when combined with use of FID to determine first the number of carbons in a hydrocarbon and then identify the chemical compound.

# Chapter 2

# EXPERIMENTAL

## 2.1 Plant material

Root samples were collected in the fall because roots are most potent after flowering season and seeds are ready to plant (Philip, 2005). In fall 2005, oshála (*L. grayi*) was collected from Bear Creek Campsite at Gold Lake, Plumas County, California (elevation 6411', 39° N, 120° W) and identified by Dr. Mary McCarthy Hintz from California State University, Sacramento. Two specimens were collected, one by the waterside and the other in an open meadow. The entire plant material, including the root, was collected (about 2 lbs). One plant was used for development of experimental methods and GC analysis at the California State University, Sacramento. One plant (50 g) was used for the final SAFE/BAENG extraction and GC-MS and GC-FID analysis at the United States Department of Agriculture (USDA), Emeryville, California.

To investigate the variability in the chemical make-up of *L. grayi* in different locations, another 4 plants were collected at the end of Fall 2007, again from Bear Creek Campsite, at Gold Lake. Samples were collected about ¼ mile, ½ mile, ¾ miles and 1 mile away from the first 2005 collection site. Plant material was stored in a freezer (-20 ºC) until analysis. Each plant represented a unique plant population and individual plants were used for SAFE/BAENG extraction and GC-MS and GC-FID analysis at the USDA.

For comparison, *L. porteri*, collected from Colorado in fall of 2005, was provided by renowned herbalist Christopher Hobbs. Extraction and GC-MS and GC-FID analysis was performed as for the *L. grayi* specimens.

### 2.2 Analysis at California State University Sacramento

Since *L. grayi* has never been chemically analyzed, methods of extracting, separating and identifying its constituents had to be developed based on previous research and related species, such as *L. porteri*. Much of the research for this thesis was based on developing and testing the best methods specifically for *L. grayi*.

#### 2.2.1 Solvent extractions

The root material collected from the *L. grayi* in Fall 2005 and from *L. porteri* was processed at California State University, Sacramento, where it was cut, crushed, and ground with a mortar and pestle until the plant material was almost granulated. About 20 g of root material was pulverized at a time for each experiment. Once the plant roots were processed, 10 g was added to a 150 mL erlenmeyer flask containing either 10 mL or 25 mL of 95 % ethanol, n-hexane, or dichloromethane, totaling 6 flasks. Each flask was shaken 2 times a day and allowed to settle overnight for a period of about 2 days. Then each mixture was filtered through a 3 mm Whatman filter paper, pre-rinsed (with its respective solvent) and dried overnight with anhydrous sodium sulfate. The above procedure was repeated two to four times for each plant used for method development.

### 2.2.2 Soxhlet extraction

The soxhlet extraction protocol was based on the procedure described in Chemical Analysis and Testing Task Laboratory Analytical Procedure (Ehrman, 1994) and performed in a chemical safety hood. Two to three glass-boiling beads were added to a 250 ml collecting round bottom receiving flask, which was then weighed. *L. grayi* root (1 g) was rolled in a 20 mg Kim wipe and placed into a 10 µm extraction thimble. A small glass rod was used to assist in the transfer of the root in the Kim wipe into the thimble. The thimble was inserted into the soxhlet extraction flask and the lower end of the extraction flask was connected to the receiving flask. Glass wool was used to plug the top to prevent sample and solvent loss during extraction (**Figure 6**). The upper end of the receiving flask was connected to a water condenser that was clamped in place over a hot plate. The receiving flask rested on a rounded block that lay on a hot plate. For each plant sample, 10 mL, 15 mL or 20 mL of hexane (used for 2 pretest runs) or ethanol (used for 2 pretest runs) was poured into a 50 mL round-bottom flask as the reflux solvent. The heating mantle evaporated and condensed the solvent at a rate of 1 drop/2 sec at 60 ˚C. The extraction continued for 6 hours a day for 4 days. Solids were filtered by vacuum filtration through a Whatman Grade 2 (24 cm) filter paper and then the filtrate was allowed to sit at room temperature for 24 hrs. The solvent then was removed by rotary evaporation and the flask was weighed again to determine the weight of the dried extract.

### 2.2.3 Vacuum distillation

Vacuum distillation was carried out using a distillation apparatus with a 150 mL beaker filled with 50 mL of distilled water on a hot plate with thermometer measuring the temperature of the water (104 ˚C). The 50 ml round-bottom flask was filled with 20 mL of *L. grayi* or *L. porteri* extract in n-hexane and sealed with a distillation head submerged in the 150 mL beaker. The distillation head was clamped to a condenser with two holes for tubing where water can enter and exit. At the end of the condenser was a fitting connected to a vacuum line and to a 20 mL side-arm receiving flask containing water to collect the distillate (**Figure 7**). Since the distillate could be clear and colorless oil, it was occasionally checked for oily drops by catching some distillate in a small beaker. Then the water/distillate mixture was poured from the receiving flask into a separatory funnel and the side-arm receiving flask rinsed with 10 mL of methylene chloride using a pasteur pipette. Finally, the funnel was shaken, allowed to settle for 30 min and the methylene chloride layer was collected in a small Erlenmeyer flask. The aqueous layer was extracted with two 10 mL portions of methylene chloride. Residual water was removed using sodium sulfate to the combined methylene chloride extracts until it remained granular. Then it was filtered through a tiny piece of cotton. Rotory evaporation was used to remove the solvent from the filtrate and the dried filtrate weighed.

### 2.2.4 Gas chromatography-mass spectrometry (GC-MS)

An HP model 5890 gas chromatograph coupled with a mass spectrometer (GC-MS) was used on a 5 % phenylsilicone stationary phase in a fused silica capillary column (30 m X 0.25 mm i.d., 1 μm; Restek). The carrier gas was helium at a flow rate of 1.0 ml/min. The data acquisition system was controlled by MS ChemStation (Agilent Technologies). The column temperature program was 40 °C for 1 min, 20 °C/min to 150°, 30°C/min to 280°C, hold 2 minutes. Electron impact MS was used for detection. 1-2 μL were used for each analysis.

## 2.3 United States Department of Agriculture (USDA) Analysis

### 2.3.1 Chemicals

Diethyl ether was freshly distilled before each extraction through a 60 cm long Pyrex column packed with glass helices then stored in the dark after addition of 1-2 ppm of antioxidant 330 (1,3,5-trimethyl-2,4,6-tris-[3,5-di-tert-butyl-4-hydroxybenzyl]-benzene; Ethyl Corporation, Richmond, Virginia). Standards were from the USDA.

### 2.3.2 Extraction

The first two analyses performed were of one *L. grayi* plant root sample (about 100g) and one *L. porteri* plant root sample (about 200g) that were both collected in Fall 2005. A later analysis was done with four *L. grayi* root samples (Pop1: 92.8g, Pop2: 57.2g, Pop3: 37.3g, Pop4: 58.42g) collected in Fall 2007. All samples were crushed with a mortar and pestle under liquid nitrogen. The material for each population was divided into equal portions and added to two 250 mL Pyrex glass bottles with Teflon-lined screw

caps. Approximately 125 mL of ether was added to each bottle. The bottles were covered with aluminum foil and were shaken every 2 h throughout the daytime for 4-5 days. The extract was filtered through pre-rinsed (ether) filter paper (ED fluted filter paper, grade 513, 24 cm diameter, Eaton-Dikeman, Mount Holly Springs, Pennsylvania). The extract was dried overnight over anhydrous sodium sulfate (previously heated to 150 °C for several hours to remove volatiles).

### 2.3.3 Solvent assisted flavor extraction (SAFE) distillation

The solvent assisted flavor evaporation (SAFE) apparatus (**Figure 8**: Engel *et al*, 1999) was used on the extract through high vacuum distillation (<0.01 Pa ). The SAFE apparatus had a distillation flask (500 mL) that was heated to 40°C using a circulating water bath. The extract was added to the dropping funnel. Then the receiving flask and the safety-cooling trap of the SAFE apparatus were cooled with liquid nitrogen. After, the extract in the dropping funnel was added in small continuous increments into the distillation flask over a 30 min time period. Concentration of the distillate was done by a Vigreux column (15 x 1 cm) and water bath at 40° C. Finally, the extract underwent GC-FID and GC-MS analyses.

### 2.3.4 Gas chromatography-mass spectrometry (GC-MS)

The GC-MS machinery comprised of an Agilent Technologies 6890 gas chromatograph affixed to an Agilent Technologies 5973 Network MSD (Agilent Technologies, Palo Alto, CA), using a 60 m X 0.25 mm (i.d.) DB-1 MS fused silica capillary column ($d_f$ = 0.25 μm; J&W Scientific, Folsom, California). During analysis,

the GC oven was programmed from 30 °C (4 min isothermal) to 200 °C at 2 °C/min (final hold at 200 ˚C for 35 min) with the carrier has helium (head pressure of 22 psi). The injector, transfer line, ion source and quadrupole temperatures were 180 °C, 200 °C, 170 °C and 130 °C. Finally, the mass spectrometer was set to electron impact mode with an ionization voltage of 70 eV.

### 2.3.5 Gas chromatography-flame ionization (GC-FID)

The GC-FID machinery comprised of a Hewlett-Packard (Avondale, PA) 6890 gas chromatograph affixed to a flame ionization detector (FID), using A 60 m X 0.32 mm i.d. DB-1 ($d_f$ = 0.25 □m; J&W Scientific, Folsom, California) fused silica capillary column. During analysis, the oven temperature was programmed from 30 °C (4 min isothermal) to 200 °C at 2 °C/min (final hold 200 ˚C for 25 min) with the carrier gas helium of a linear velocity of 38.3 cm/s (30 °C). Split injections were 1:20.

### 2.3.6 GC-FID & GC-MS analysis scheme

Below is a flow chart (**Figure 13**) for the identification of constituents from both *L. grayi* and *L. porteri*. The GC-FID produced data that were used to calculate the retention index (RI) from authentic standards, which were then compared to RI values given in the Kovats library of standards. The GC-MS data also included retention times which were used to calculate its own RI values that were compared to the Wiley and Adams library. RI values and retention times from GC-FID were then compared with RI values and retention times from GC-MS (**Table 1 & 2**). Finally the MS (mass spectra) produced

from the GC-MS were compared to a library MS-3 and PMB, which resulted in the identification of all compounds (**Tables 1 & 2**).

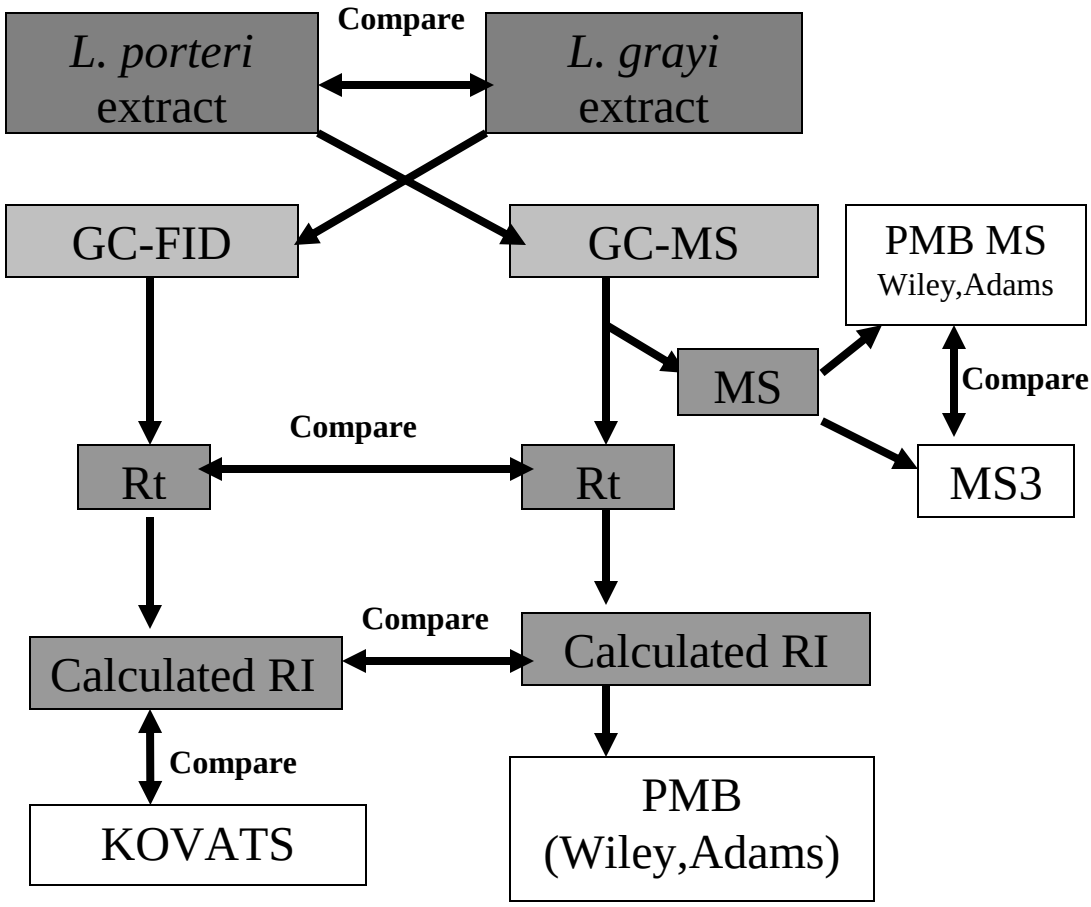


**Figure 12**: Comparison pathway for the identification of ***L. grayi*** by GC-MS/GC-FID

### 2.3.7 Identification of volatiles: libraries

Each volatile constituent was identified by comparing the component's mass spectrum (MS) and experimental retention index values (RI) with that of an authentic reference standard. When standards were not available, tentative identifications were assigned based on mass spectra and retention indices reported in Wiley Registry of Mass Spectral Data, 6$^{th}$ Edition (John Wiley & Sons, Inc., Hoboken, NJ), MassFinder 3 (Dr.

Hochmuth Scientific Consulting, Hamburg, Germany) and Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th Ed. (Adams, 2007).

**2.3.8 Identification of volatilees: Confirmation of number of carbons**

The retention system proposed by Kováts (1958) was utilized for comparison. The number of carbons was estimated by using the results from the FID for each plant to calculate the Retention Index (Formula 1). Retention time of each peak was used with the time range between the elution of each carbon group number (C4, C5, C6, C7 up to C20) resulting in the following calculation. Standards (from USDA) were used to determine the carbon group numbers.

$$\text{Report Index} = \frac{[\text{Log (Rtm}- \text{tmd})]-[\text{Log (Cx-tmd)}]}{[\text{Log (Cy-tmd)}]-[\text{Log (Cx-tmd)}]} \times 100$$

Report Index Final = Repor Index + (C x 100)

Rtm= Retention time of each peak

Tmd= Drag time (2.62 min)

Cx = Starting carbon retention time

Cy = Ending carbon retention time

C = # carbons

The retention time is the time each anlayte elutes off the column. The drag time is the dead time it took for any analyte to start eluting off the column. Cx is the starting time of the first peak to elute in the carbon group (ex. C4) and Cy is the ending time of the final peak to elute for the carbon group which is equivalent to the starting point of the next carbon group (eg. C5). C is the number of carbons in the molecule; for instance C5 is equivalent to a 5-carbon molecule.

# Chapter 3

# RESULTS AND DISCUSSION

## 3.1 Simple extraction chromatography

Initially, simple extractions were executed to find the best method for *L. grayi*. Extractions were first performed on *L. porteri* because it was easily obtainable and is well characterized compared to *L.grayi*. The three extraction solvents tested were hexane, 95% ethanol, and dichloromethane. These extraction solvents were chosen because the solvents are abundant and inexpensive. According to the gas chromatograms depicted in **Figures 13, 14,** and **15** below, there is a difference in the efficacy of extraction with theses three solvents.

The extraction with hexane yielded the most compounds that could be seen by GC-MS (**Figure 13**). The first three peaks (4.9, 5.4, 6.1 min.) are solvent peaks (hexanes). Of the seven other peaks seen in the chromatograph, four compounds were completely identified in the hexane extract; α-pinene, sabinene, β-pinene and ocimene, with retention times of 12.5, 13.0, 13.3 and 13.6 min., respectively. These compounds were identified by comparing mass spectra to a mass spectral library of known compounds using the Benchtop PMB program. The hexane extracts (four runs/plant) produced the most visible peaks with the highest intensity, the least broadening, and minimal tailing. GC-MS of the 95% ethanol extract (**Figure 14)** yielded three major peaks, with two of the peaks identified as β-pinene and δ-3-carene (retention times 9.8, 10.2 min). Clearly, this extraction method is not an acceptable extraction method because the peaks were too

small to clearly identify more compounds. The dichloromethane extraction was the least productive of all three extraction methods and produced only two peaks (5.1, 6.3 min) that were too low in intensity (4000) to be able to identify any compounds, but were assumed to be due to the solvent.

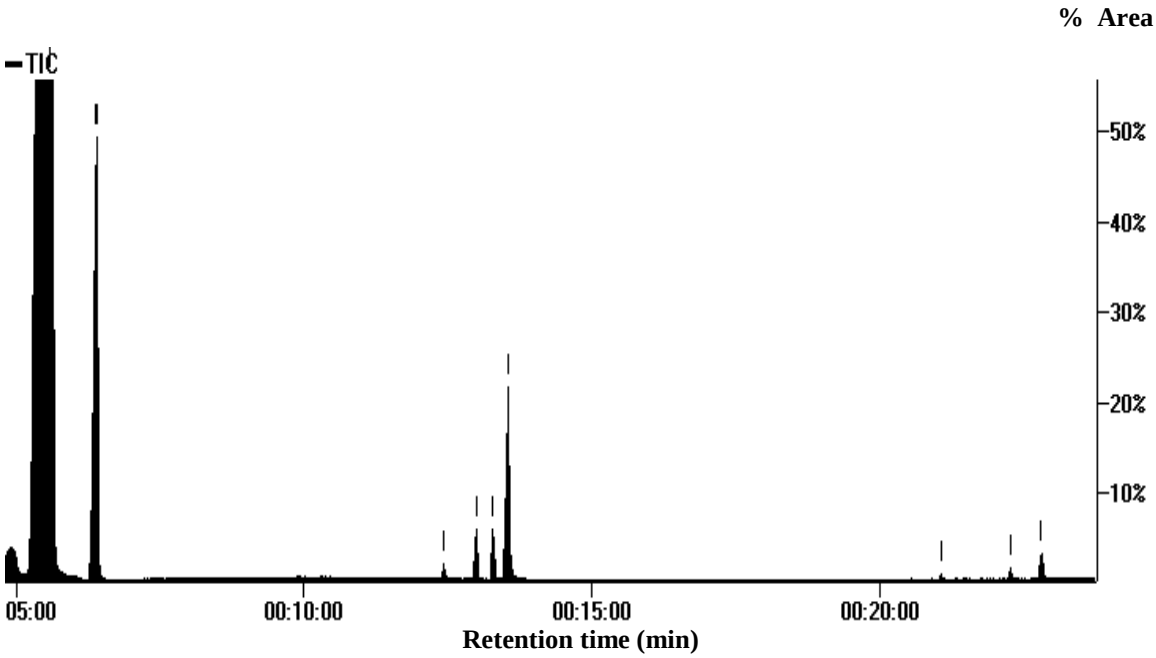


**Figure 13**: GC-MS chromatogram of hexane extraction of *L. porteri* roots

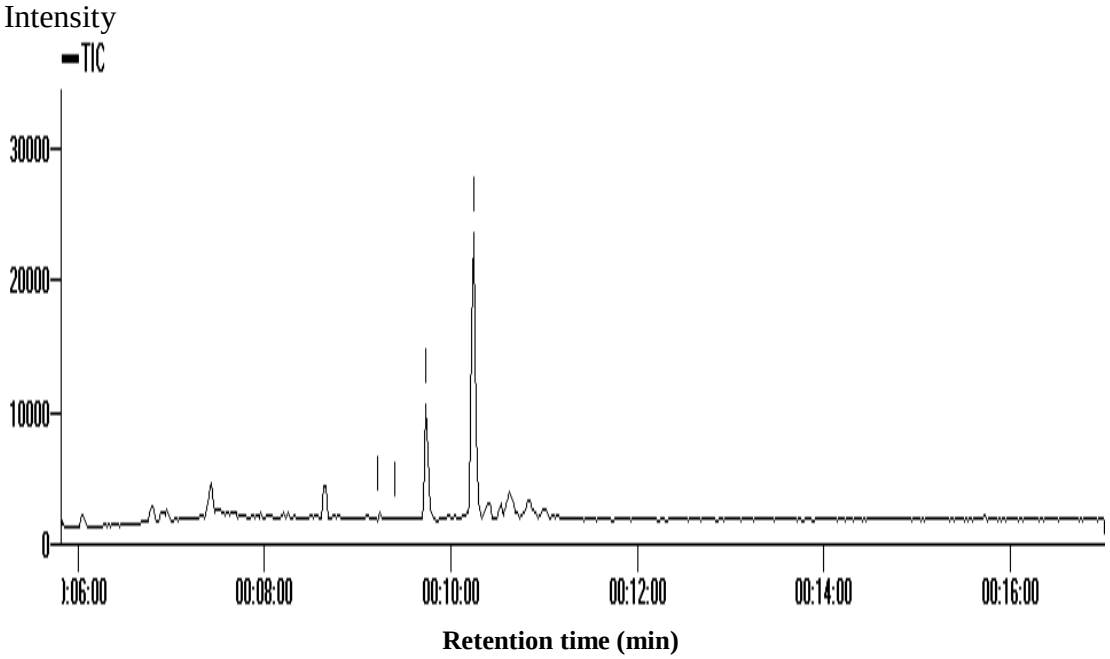


**Figure 14**: GC-MS chromatogram of 95% ethanol extraction of *L. porteri*

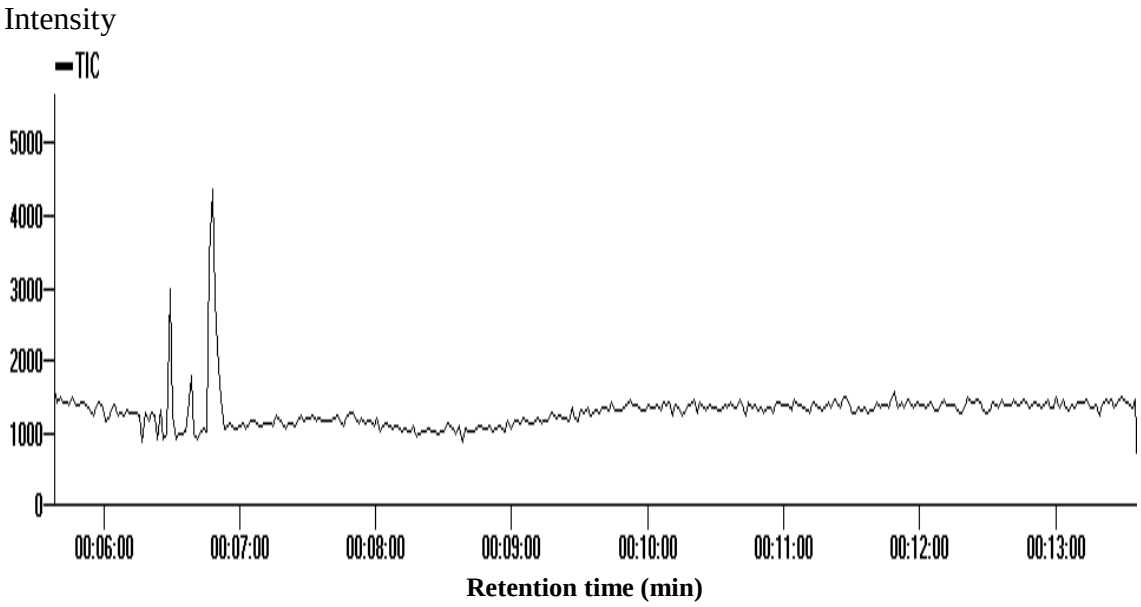


**Figure 15**: GC-MS chromatogram of dichloromethane extraction of *L. porteri*

Of all the simple extraction methods (hexane, ethanol, and dichloromethane), hexane proved to be best for yield and identification of chemical constituents. GC-MS of the hexane extract yielded seven peaks, compared to only three peaks for the ethanol extract and one peak for the dichloromethane extract. Out of the seven peaks from the hexane extract, four compounds were identifiable; α- pinene, β-pinene, β-ocimene, and sabinene (**Figure 13**), which are terpenes. Terpenes, which are often found in essential oils, are derived biosynthetically from units of isoprene, which are one of nature's basic building blocks. Terpenes become more complex as isoprene units polymerize and bind together (Bolmann, 1998), forming monoterpenes (2 isoprene units), sesquiterpenes (3 units), diterpenes (4 units), sesterterpenes (5 units), triterpenes (6 units), sesquaterpenes (7 units), tetraterpenes (8 units) and polyterpenes (many units), as shown in **Figure 16**. In GC-MS, pinenes, ocimene, and sabinene would be the first compounds to be eluted because they are monoterpenes, consisting of only two isoprene units, $C_{10}H_6$, therefore are the most volatile.

In terms of retention times, elution of hexane peaks ranged from 10 to 24 minutes, while only three peaks were visible from 8 to 11 minutes for the ethanol extract, and a solvent peak at 6.3 minutes for dichloromethane. The hexane extract had more peaks and a wider range of peaks, at a higher retention time range. Therefore, hexane, which is a cheap, simple, and abundant solvent, is capable of extracting more volatiles than ethanol and dichloromethane. However, since only seven peaks could be identified using simple solvent extractions, other extraction methods were explored. From these results, hexane was chosen as the solvent for other extraction methods.

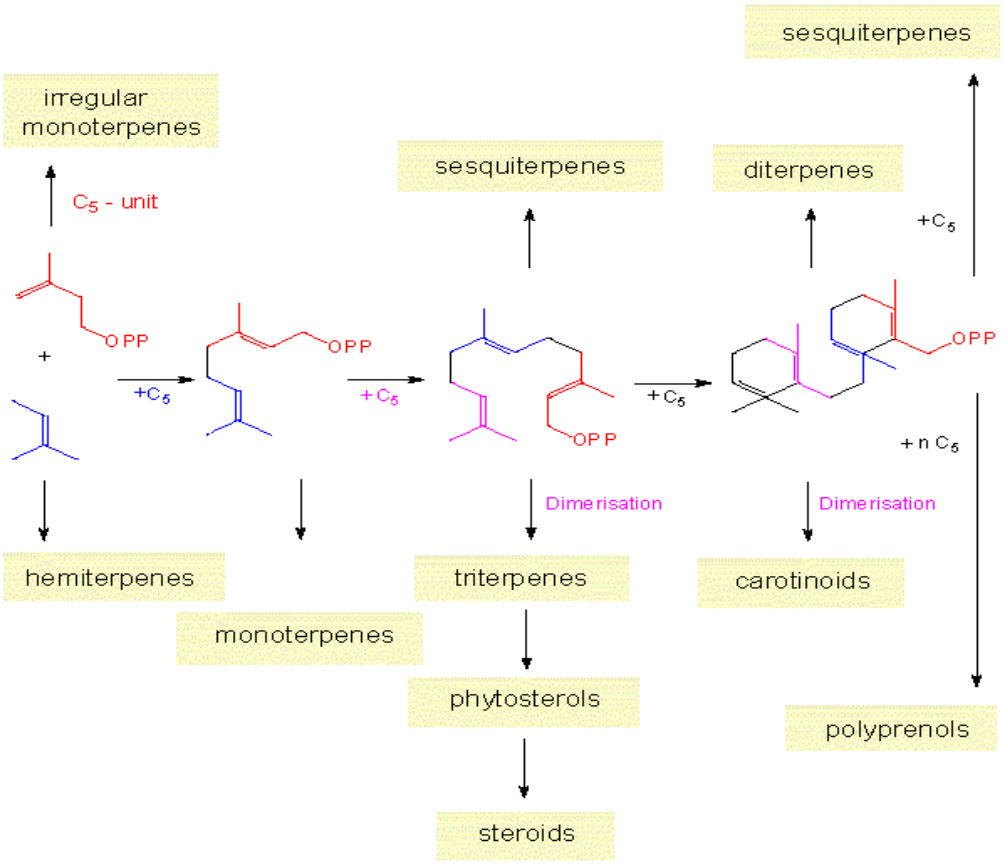


**Figure 16:** Terpene formation in plants (Bergfed, 2003)

**3.2 Soxhlet extraction chromatography**

The soxhlet extraction method (**Figures 17a, 17b & 18a, 18b**) produced dramatically better results than the simple dichloromethane, hexane, and 95% ethanol extraction methods. The chromatograph produced 49 total peaks, with 12 peaks large enough to be identifiable, for *L. porteri*. Since *L. porteri* results were successful, a preliminary test was performed with *L. grayi* roots, which resulted in 53 total peaks, with 16 peaks having matches in the PMB library, ranging from 40-90% confidence for compound identity based strictly on mass spectra (**Table 1**). Unfortunately, not every peak resulted in a reliable MS or retention time confirmation. Due to varying confidence, the MS of each peak (12 peaks for *L. porteri* and 16 for *L. grayi)* was also further analyzed visually to determine if there was an exact match between the MS of the sample compound and the MS in the database. For both *L. porteri and L. grayi*, only 7 compounds matched by retention time and MS comparison, as noted by an asterisk on **Table 1** - α-thujene, α-pinene, sabinene, β-pinene, α-phellandrene, terpinene, and β-phellandrene. To further confirm the results, standards were also run for α-thujene, α-pinene, sabinene, β-pinene, and α-phellandrene. δ-3-Carene was also run because its MS is closely related and sometimes mistaken for a pinene.

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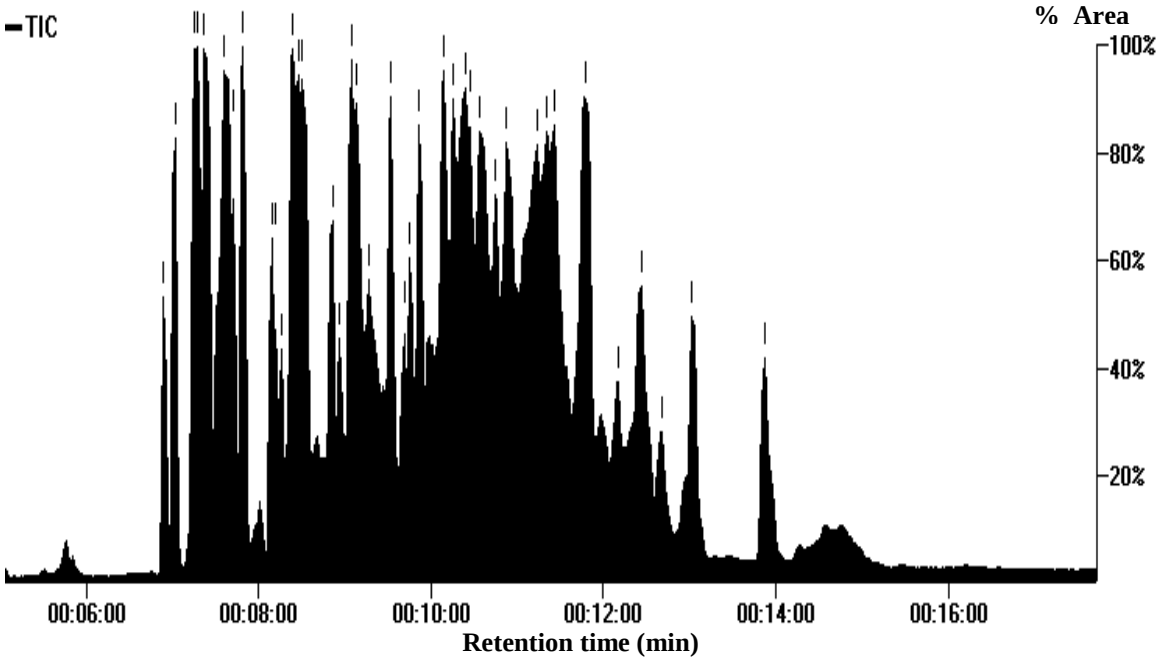


**Figure 17a**: GC-MS chromatogram of hexane soxhlet extraction of *L. porteri*

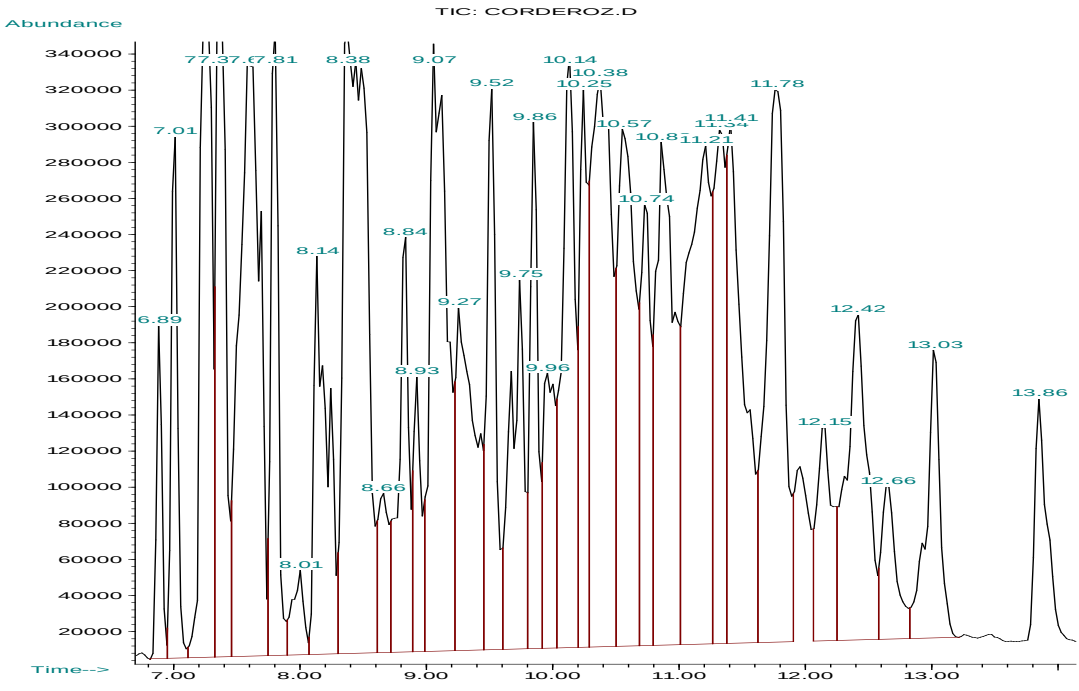


**Figure 17b**: Expanded peak verification of soxhlet extraction of *L. porteri* with hexane

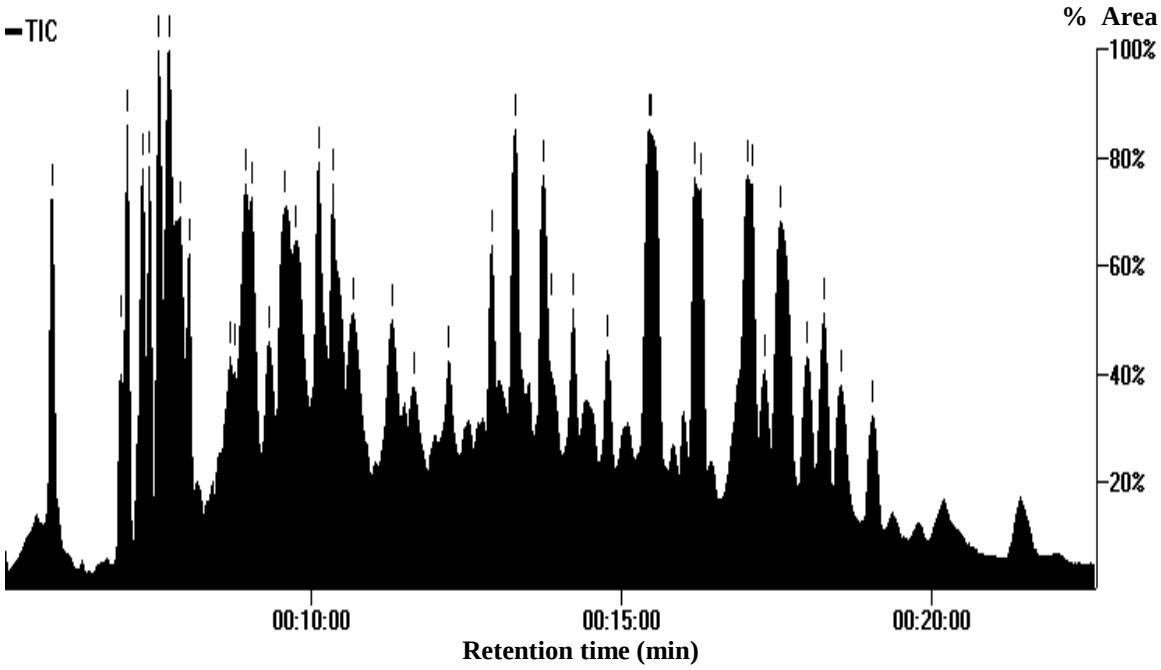


**Figure 18a**: GC-MS chromatogram of hexane soxhlet extraction of *L. grayi*

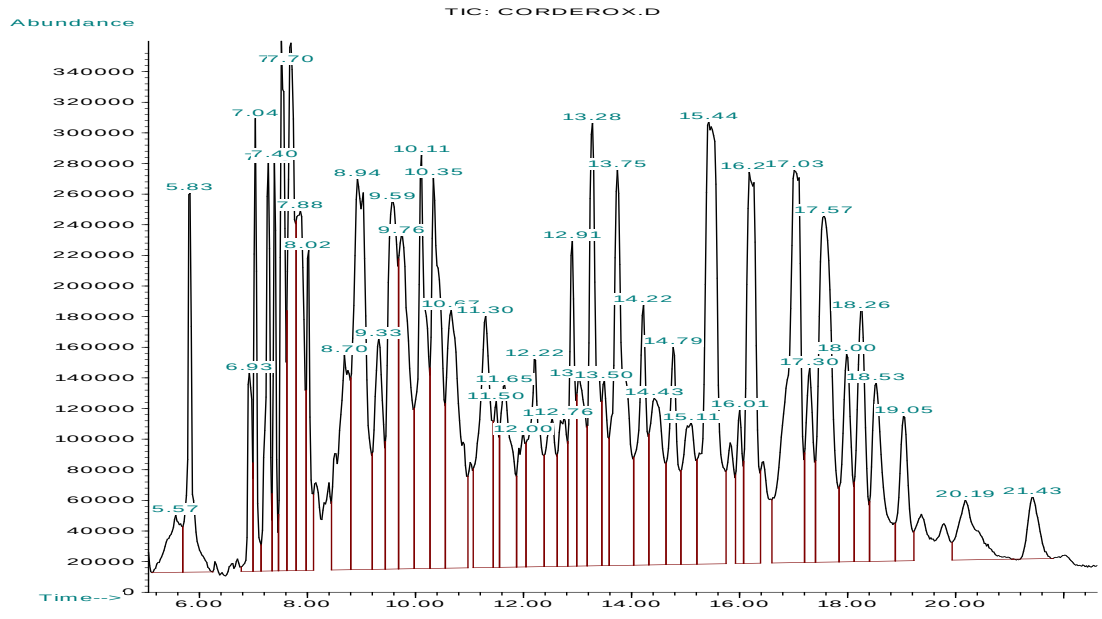


**Figure 18b**: Expanded peak verification of soxhlet extraction of *L. grayi* with hexane

**Table 1:** Compound % confidence identifiable by PMB library of *L. grayi* and *L. porteri* by soxhlet extraction. The identity of compounds with asterisks was confirmed by visual analysis.

| Retention Time (min) | % Identification | Retention time (min) | % Identification | Retention time (min) | Possible Compound |
|---|---|---|---|---|---|
| ***L. porteri*** | ***L. porteri*** | ***L. grayi*** | ***L. grayi*** | **Standards** | **Possible Compound** |
| 5.76 | 81% | 5.83 | 80% | 5.93 | α-thujene * |
| 7.01 | 83% | 7.01 | 87% | 6.926 | α-pinene * |
| 7.25 | 95% | 7.25 | 91% | 6.73, 7.06 | sabinene * |
| 7.36 | 94% | 7.36 | 87% | | β-pinene * |
| | | 7.5 | 79% | 7.365 | 3-carene |
| 7.61 | 90% | 7.65 | 91% | 7.234, 7.453 | α-phellandrene * |
| 7.8 | 94% | 7.98 | 95% | | terpinene * |
| 8 | 70% | | | | 2-carene |
| 8.44 | 50% | 8.46 | 30% | | methyl ethyl cyclopentene |
| 9.52 | 98% | 9.8 | 89 % | | β-phellandrene * |
| 9.87 | 43% | | | | β-myrcene |
| | | 10.05 | 98% | | methanoazulene |
| | | 10.29 | 56% | | fluoranthene |
| 10.38 | 70% | | | | azulene |
| 10.88 | 76% | | | | apiol |
| | | 11.12 | 43% | | caryophyllene |
| | | 11.21 | 53% | | naphthalene |
| | | 11.74 | 86% | | pyrene |
| | | 12.79 | 74% | | dibytul phthalate |
| | | 14.64 | 72% | | α-farnesene |

In general, soxhlet is a great extraction method because it utilizes a reflux cycle system of fresh solvent that can run for days. During each cycle, a portion of the soluble components dissolves in the solvent and is concentrated further after many cycles into the distillation flask. This method is advantageous because the solvent is perfectly mixed throughout the duration of the experiment, whereas the simple extraction method allows the solvent and plant material to sit and settle along the bottom of the flask. Furthermore,

the solvent passes through the plant sample in the thimble of the soxhlet extractor, keeping the mass transfer constant and the extract concentration dependent on the time of the extraction.

Compared to the simple solvent extraction methods (hexane, ethanol and dichloromethane), the soxhlet process extracted many more compounds - 49 visible peaks for *L. porteri* and 53 visible peaks for *L. grayi*. Soxhlet extraction was able to extract 10 times as much of the volatiles than even the best simple solvent extractions. The disadvantage of simple solvent extraction is the amount of solvent being used, especially during large-scale extractions, and the time it takes, hours to days, with no possibility of increasing the yield of the extraction process (compared to soxhlet extraction). A disadvantage of the soxhlet extraction is that the heat used during cycling may cause evaporation and deterioration of the compound during long periods of time. However, even with soxhlet extraction, the percentage of identifiable compounds is low, and only 16 compounds were identified using the same identification parameters as the solvent extraction (with MS's very closely matching MS's in the PMB library). Therefore, comparison of retention times of *L. grayi* constituents to those of *L. porteri* constituents and comparison to other MS libraries were used to identify more of the compounds in the soxhlet extract of *L. grayi*.

In regards to the differences between plant root samples (*L. porteri* vs. *L. grayi*), the two chromatograms are very similar, but *L. grayi* has more peaks than *L. porteri*, and the retention time of the last compound eluted is 30 min, while the elution of compounds from *L. porteri* ends around 15 min. The chemical composition of *L. porteri* has already

been reported in the context of antibacterial research (Cegiela-Carlioz et al, 2005; **Table 2**), but not that of *L. grayi*. Since both plants derive from the same genus and *L. grayi* contains more compounds than *L. porteri,* knowledge of the chemical composition of *L. grayi* will be useful for antibacterial and comparative secondary metabolite research. As mentioned, *L. porteri* essential oils are found to be rich in monoterpenes such as pinenes, thujene, phellandrenes, and phthalides such as ligustilide and butyldiene pthalides (Agnihori, 2004). As shown in **Table 1**, the same can be said for soxhlet extracts of *L. porteri* and *L. grayi*. Soxhlet extraction provided confirmation of the monoterpenes (α-pinene, β-pinene, and sabinene) from the simple extractions as well as identifying other monoterpenes such as α-thujene, phellandrenes, and terpinene., showing 60% similarity between the two plants, and identification of many more monoterpenes.

Unfortunately, sesquiterpenes in *L. porteri* and *L. grayi* were not identifiable by MS because there were too many overlapping peaks; the constraints of the older Sac State GC machinery and soxhlet extraction did not allow for good peak separation or high peak intensity at longer elution times (**Figure 17b, 18b**). Sensitivity to larger carbon molecules is low in GC because of their lack of volatility, and only 7 compounds were identifiable in the soxhlet extraction. Therefore, another extraction method, SAFE-BAENG method, was utilized for better qualitative and quantitative results.

**Table 2**: RI and % essential oils found in extracts of the roots of *L. porteri* (Cegiela-Carlioz et al, 2005)

| Compounds | Retention Indices | Essential Oil (%) | Dichloromethane extract (%) |
|---|---|---|---|
| α-thujene | 926 | 0.5 | |
| α-pinene | 942 | 0.1 | |
| sabinene | 973 | 0.4 | 0.1 |
| β-pinene | 978 | 0.3 | |
| myrcene | 997 | 0.1 | |
| α-phellandrene | 1003 | 0.3 | 0.1 |
| α-terpinene | 1017 | 0.2 | |
| ρ-cymene | 1018 | 0.9 | 0.2 |
| β-phellandrene | 1026 | 0.4 | |
| limonine | 1027 | 0.1 | 1.8 |
| γ-terpinene | 1056 | 0.3 | 0.2 |
| cis thujone | 1103 | 0.03 | |
| 1,3,8 mentharlene | 1112 | 0.1 | |
| α-phellandrene 1,2 epoxide | 1115 | 1.9 | 0.6 |
| sabinol (3)* | 1132 | 3.3 | |
| ibenunene | 1161 | 2.1 | 1.5 |
| terpinene 4-ol | 1170 | 0.8 | 0.1 |
| α-terpine oil | 1181 | 0.1 | |
| thymol methyl ether | 1228 | 0.05 | 0.4 |
| carvacryl methyl ether | 1254 | 0.9 | 0.4 |
| isothujyl acetate | 1273 | 0.2 | 0.1 |
| trans pinocarveyl acetate | 1273 | 0.1 | |
| bomyl acetate | 1284 | 0.2 | 0.9 |
| sabinyl acetate | 1287 | 56.6 | 34.6 |
| 4-vinylgualiacol | 1299 | 0.4 | |
| 4-terpinyl acetate | 1338 | 0.1 | 3.1 |
| α-terpinyl acetate | 1348 | 1.3 | 0.8 |
| methyl eugenol | 1389 | 1.1 | 0.5 |
| 2-5 dimethoxy-ρ cymene | 1407 | 0.3 | 0.2 |
| α-barbatene | 1413 | 0.3 | 0.4 |
| β-fenebrene | 1423 | 0.1 | 0.1 |
| widdrene | 1441 | 0.2 | 0.3 |
| β-barbatene | 1444 | 1.3 | 1.5 |
| myristicin | 1448 | 0.8 | 0.6 |
| - kessone | 1500 | 0.6 | 0.4 |

| | | | |
|---|---|---|---|
| liguloxide | 1505 | 0.5 | 0.4 |
| α-chamignene | 1507 | | 0.1 |
| elemicin | 1531 | 0.5 | 0.4 |
| (Z) 3 butylidenephthalide | 1637 | 0.8 | 0.8 |
| α-eudesmol | 1658 | 0.2 | 0.1 |
| (Z) ligustilide | 1698 | 12.9 | 39.1 |
| (E) ligustilide | 1749 | 0.2 | 0.8 |
| Identified compounds | | 88.1 | 96.2 |

### 3.3 SAFE-BAENG extraction

#### 3.3.1 Overview

The SAFE method used by the USDA Emeryville campus proved to be the best extraction method for this study. Based on this study, the advantages of the SAFE extraction method are the high yield, including excellent extraction of monoterpenes and sesquiterpenes, minimal solvent use and short extraction time. The disadvantages encountered are the expensive, unique glassware, as well as having to carefully fill the compartments with liquid nitrogen and checking the machinery continuously for more than an hour during distillation. Furthermore, it is difficult to clean the condenser, and purification can be lower because of a greater chance of environmental contamination. Another important factor for better extraction of volatiles is the freshness of a five-day frozen root versus a five month frozen root sample. Fresh root samples had a small beneficial change, yielding taller peaks than the older roots. Of all the extraction methods, SAFE extraction with the BAENG apparatus was the best extraction method due to greater extraction of most volatiles in the compound, high sensitivity, less solvent waste, and most of all, characterization of larger hydrocarbons such as sesquiterpenes. Also, there is the advantage of the USDA high performance Agilent HP 6890 GC

machinery paired with SAFE extraction that lead to better peak resolution and higher concentrations of compound characterization than Sacramento State's older HP 5890 GC coupled with the simple soxhlet extraction.

### 3.3.2 Comparison of *L. porteri* and *L. grayi* constituents

The GC-MS and GC-FID data for the first *L. grayi* sample (**Figures 19a-19e**) were compared to those of the *L. porteri* sample (**Figure 20**). Not only was the MS of each chromatographic peak compared to those of known compounds by the PMB program (Wiley and Adams library) and MS-3 program (Adams-DB library), but each peak was also compared by retention time (GC/MS) and retention index (GC-FID) to libraries of retention indexes (Adams-DB and Kovats) to confirm compound identification in both plant species (**Table 3 &4**). Comparison to gas chromatographic and mass spectral databases resulted in identification of 31 compounds in *L. grayi* (out of 154 total peaks) and 29 compounds in *L. porteri* (out of 160 total peaks),as shown in **Tables 3 and 4**. Visual verification confirmed identification of 19 compounds for *L. grayi* and 25 compounds for *L. porteri* compared through all programs. The two species had 14 identical compounds, equaling 64% similarity.

The percent composition of each compound was calculated for *L. grayi* and *L. porteri*, (**Table 5**). The most abundant chemical component in *L. porteri* was sabinyl acetate (23%) and in *L. grayi* was viridine (19%). *L. porteri* also has a high percentage of β-pinene (16%). Z-ligustilide was run as a standard (**Figure 21)** to verify one of the largest peaks found in *L. porteri*, (81.5 min, 12.9 %). Only *L. porteri* was characterized

as containing Z-ligustilide. There is a large peak at 65 min retention time (**Figure 19d**) and 85 min retention time (**Figure 19e**) that represents an unknown compound (4%) in the roots of *L. grayi*. The presence of this apparently novel compound led to an investigation by the USDA, using NMR to identify novel compounds found in *L. grayi*. Cool et al (2010) found that *L. grayi* contained numerous irregular sesquiterpenoids, including the known compounds acyclic sesquilavandulol, sesquilavandulyl aldehyde, thapsane, and epithapsane, and eight novel compounds.

In summary, twenty-five compounds were identified in *L. porteri*, and nineteen compounds in *L. grayi*, with a significant amount of yield for each plant. Most (95%) of the identified compounds in both plant roots are terpenes As shown in **Table 3**, 64% of the constituents are the same between the species, (14 compounds in common). Most of them are monoterpenes, and a few are sesquiterpenes. Both plants have a significant amount of β-pinene. Z-ligustilide is only present in *L. porteri*, but the major compound in *L. porteri* is sabinyl acetate (23% of volatiles) and in *L. grayi* is γ-terpinene (18%).

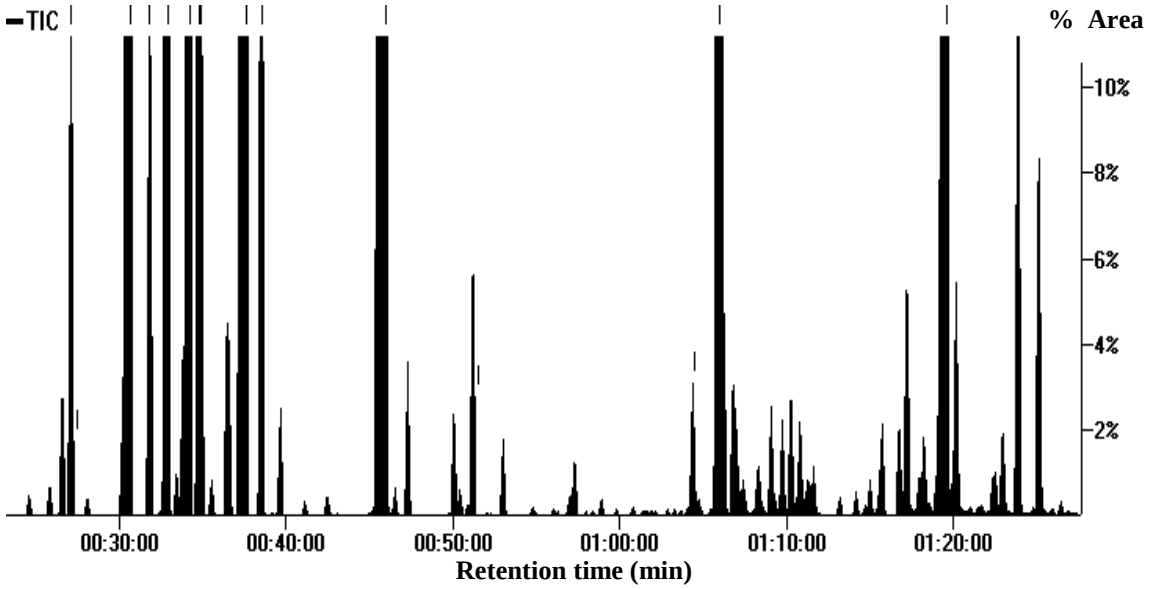


**Figure 19a**: Gas chromatogram of SAFE extract of *L. grayi* population 1

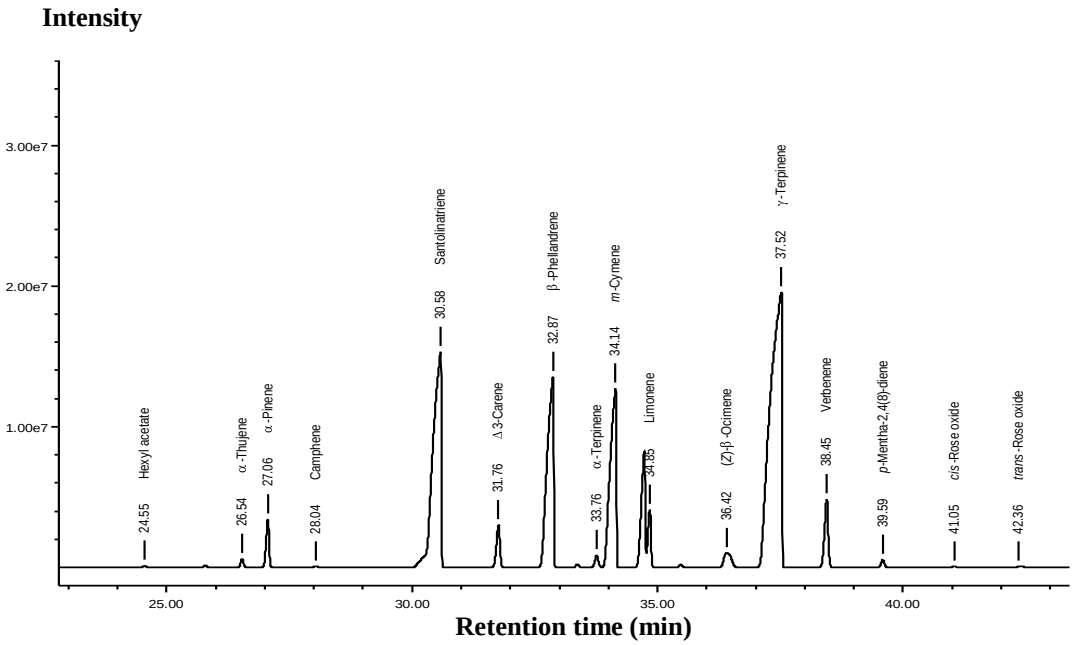


**Figure 19b**: Expanded gas chromatogram of SAFE extract of *L. grayi* pop. 1 (25-45 min)

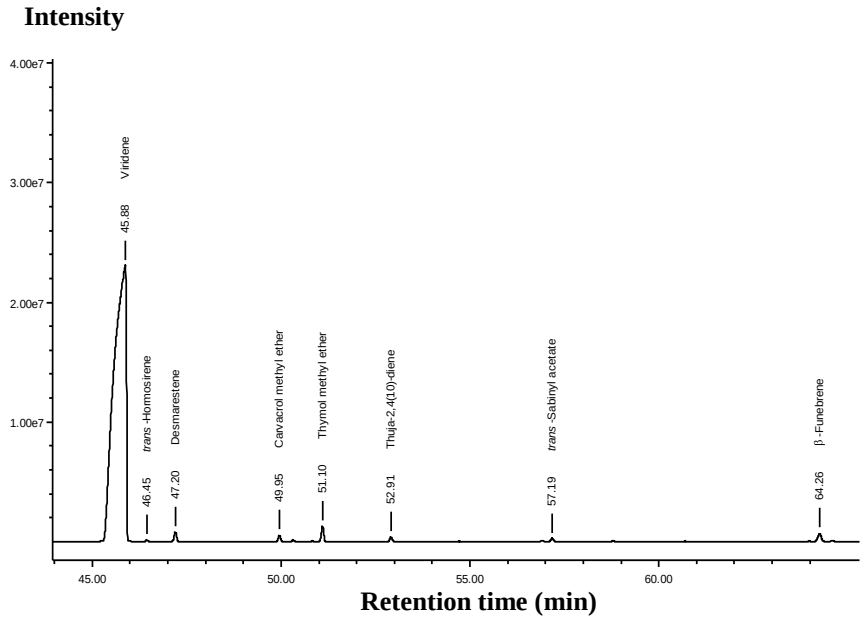


**Figure 19c**: Expanded gas chromatogram of SAFE extract of *L. grayi* pop. 1 (45-65 min)

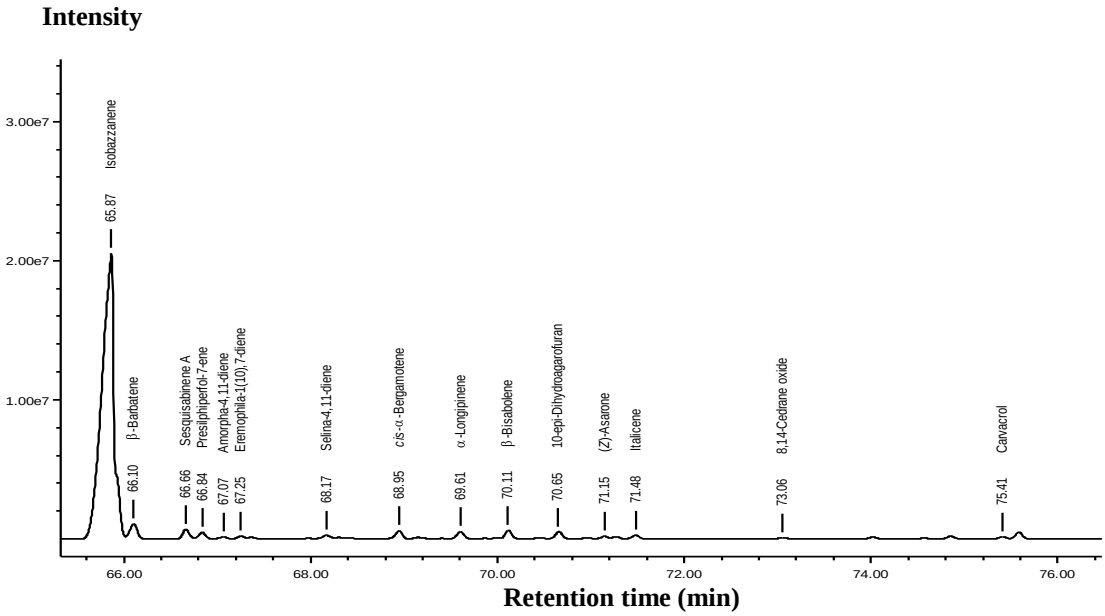


**Figure 19d**: Expanded gas chromatogram of SAFE extract of *L. grayi* pop. 1 (60-75 min)

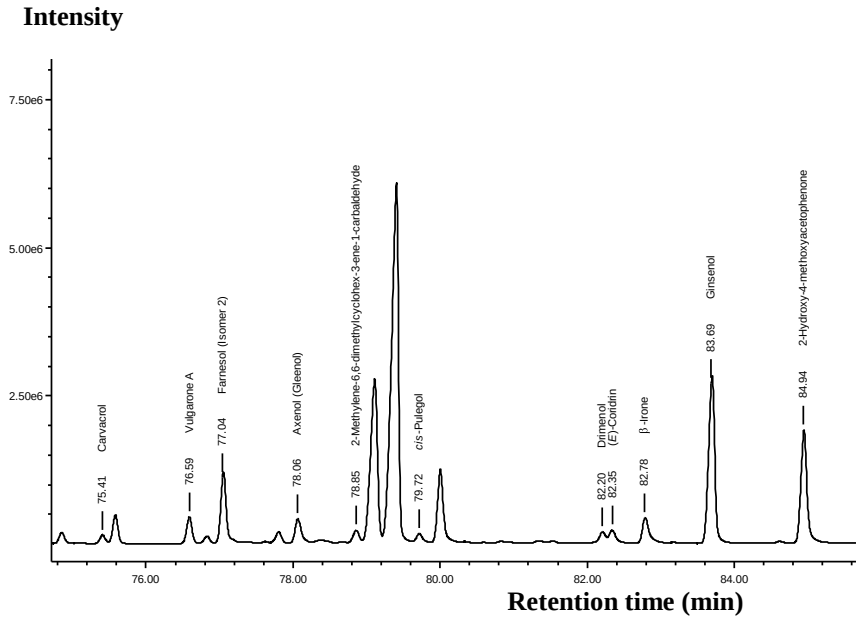


**Figure 19e**: Expanded gas chromatogram of SAFE extract of *L. grayi* pop. 1 (75-85 min)

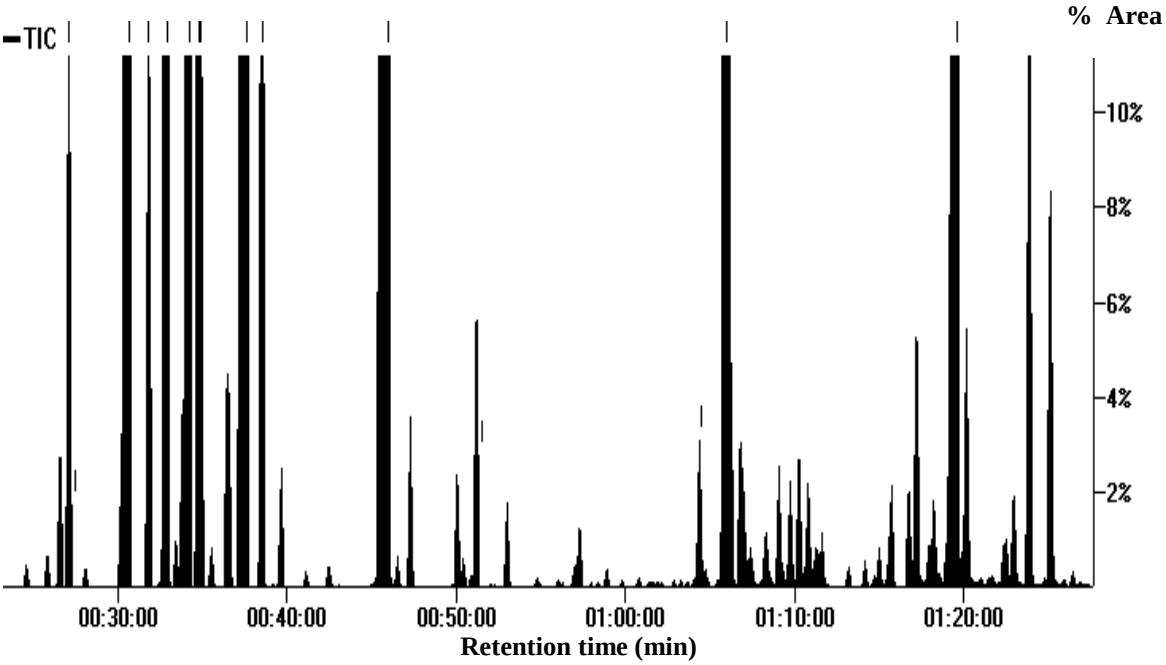


**Figure 20**: Gas Chromatogram of SAFE extract of *L. porteri*

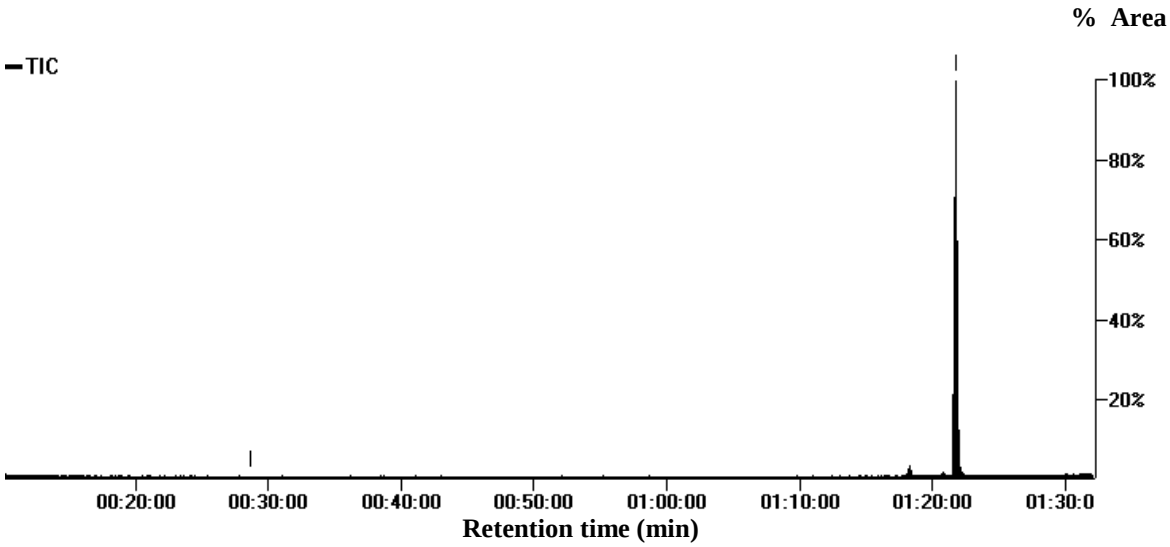


**Figure 21**: Gas chromatogram of z-ligustilide standard

**Table 3**: Comparison of retention time (Rt) and retention index (RI) of compounds found in *L. porteri*

| Compound (PBM) | Compound (MS-3) | GC/MS Rt (min) | DB-1 Calculated FID | RI MS-3 | DB-1 (NEW) | AI (R.P. Adams-DB-5) | Standard of DB-1 (Kovask | Compound Confirmed |
|---|---|---|---|---|---|---|---|---|
| α-thujene | α-thujene | 26.507 | 923 | (RI 924, | 922 | 924 | 922 | α-thujene |
| α-pinene | sabinene | 27.048 | 929 | (RI 930, | 964 | 932 | 929 | α-pinene |
| camphene | | 28.0 | 940 | (RI 940 | | 946 | 941 | Camphene |
| β-pinene | β-pinene | 30.476 | 972 | (RI 970, | 968 | 974 | 968 | β-pinene |
| β-myrcene | | 31.776 | 986 | (RI 985, | | 988 | 981 | β-myrcene |
| α-terpinene | α-terpinene | 33.739 | 1005 | (RI 1008, | 1008 | 1014 | 1008 | α-terpinene |
| ρ-cymene | m-cymene | 34.109 | 1012 | (RI 1012, | 1010 | 1012 | 1010 | m-cymene |
| β-phellandrene | β-phellandrene | 34.615 | 1017 | (RI 1018, | | 1025 | 1018 | β-phellandrene |
| limonene | limonene | 34.807 | 1019 | (RI 1021, | 1020 | 1024 | 1020 | limonene |
| (Z) β-ocimene | (Z) β-ocimene | 35.470 | 1031 | (RI 1029, | 1026 | 1032 | 1026 | (Z) β-ocimene |
| (E) β-ocimene | | 36.333 | 1041 | (RI 1039, | | 1044 | 1037 | (E) β-ocimene |
| γ-terpinene | γ-terpinene | 37.229 | 1051 | (RI 1050, | 1048 | 1054 | 1048 | γ-terpinene |
| p-mentha-1,5,8-triene | p-mentha3-8diene | 38.351 | 1063 | (RI 1064, | | 1068 | | ρ-mentha3-8diene |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| α-thujone | α-thujone | 40.034 | 1081 | (RI 1085, | | | 1084 | α-thujone |
| cis-thujone | β-thujone | 40.910 | 1091 | (RI 1095, | 1095 | | 1095 | β-thujone |
| α -phellandrene epoxide | trans sabinol | 43.058 | 1119 | (RI 1122, | 1122 | 1137 | | α-ph epoxide, trans sabinol |
| 2,4,6,8-undecatetraene | viridene | 45.898 | 1159 | (RI 1158, | 1159 | 1163 | | viridene |
| 4-terpineol | terpinene-4-ol | 46.247 | 1162 | (RI 1163, | | 1174 | 1159 | 4-terpinenol |
| thymol methyl ether | | 50.332 | 1213 | (RI 1216, | | 1232 | | thymol methyl ether |
| carvacrol methyl ether | carvacrol methyl ether | 51.112 | 1225 | (RI 1227, | | 1241 | | carvacrol methyl ether |
| bornyl acetate | | 54.259 | 1265 | (RI 1270 | 1272 | 1287 | 1268 | bornyl acetate |
| sabinyl acetate | trans sabinyl acetate | 55.059 | 1281 | (RI 1282, | | 1289 | 1268 | trans sabinyl acetate |
| | trans carvyl acetate | 57.933 | 1318 | (RI 1322, | | 1339 | 1342 | trans carvyl acetate |
| α-terpinyl acetate | α-terpinyl acetate | 58.877 | 1331 | (RI 1336, | 1342 | 1346 | 1331 | α-terpinyl acetate |
| nerol | cis carvyl acetate | 59.514 | 1341 | (RI 1345, | | 1420 | 1315 | cis carvyl acetate |
| ` | α-funebrene | 61.443 | 1365 | (RI 1373, | | 1380 | | 2epi-α-funebrene |
| β-funebrene | β-funebrene | 64.043 | 1409 | (RI 1410, | | 1413 | | β-funebrene |
| calarene/+-β – gurjunene | sesquisabinene | 66.089 | 1439 | (RI 1441 | | 1457 | 1430 | sesquisabi |
| diepi-α--cedren | β-selinene | 68.285 | 1475 | (RI 1473, | 1450 | | 1480 | β-selinene |
| Z-ligustilide | Z-ligustilide | 81.8 | 1682 | | | | 1682 | Z-ligustilide |

**Table 4**: Comparison of retention time (Rt) and retention index (RI) of compounds found in *L. grayi.*

| Compound (PBM) | Compound (MS-3) | GC/MS Rt (min) MS-3 | Calculated RI from DB-1 GC-FID | AI (R.P. Adams - DB-5) | standards of DB-1 GC-MS (Kovats) | Compound (Kovats Confirmed) |
|---|---|---|---|---|---|---|
| α-thujene | | 26.5 | 923 | 924 | 922 | α-thujene |
| α-pinene | α-pinene | 27.1 | 929 | 932 | 929 | α-pinene |
| camphene | camphen | 28.0 | 941 | 946 | 941 | camphene |
| β-pinene | β-pinene | 30.6 | 968 | 974 | 968 | β-pinene |
| β-myrcene | β-myrcene | 31.9 | 981 | 988 | 981 | β-myrcene |
| α-phellandrene | | 32.9 | 996 | 1002 | 996 | α-phellandrene |
| δ-3-carene | δ-3-carene | 33.4 | 1004 | 1008 | 1004 | 3-carene |

| | | | | | | |
|---|---|---|---|---|---|---|
| α-terpinene | α-terpinene | 33.8 | 1008 | 1014 | 1008 | α-terpinene |
| m-cymene | m-cymene | 34.1 | 1010 | 1020 | 1010 | m-cymene |
| β-phellandrene | | 34.7 | 1018 | 1025 | 1018 | β-phellandrene |
| limonene | limonene | 34.8 | 1020 | 1024 | 1020 | limonene |
| (Z) β-ocimene | (Z) β-ocimene | 35.5 | 1026 | 1032 | 1026 | (Z) β-ocimene |
| (E) β-ocimene | | 36.4 | 1037 | 1044 | 1037 | (E) β-ocimene |
| γ-terpinene | γ-terpinene | 37.5 | 1048 | 1054 | 1048 | γ-terpinene |
| α-terpinolene | α-terpinolene | 39.6 | 1077 | | | α-terponelen |
| cis-rose oxide | cis-rose oxide | 41.1 | 1096 | | 1096 | cis-rose oxide |
| trans-rose | trans-rose | 42.4 | 1110 | 1112 | | trans-rose |
| viridene | viridene | 45.9 | 1132 | 1163 | 1167 | viridene |
| undeca-1(E,E,Z)-3,5,8-triene | trans-hormosirene | 46.4 | 1172 | | | trans-hormosirene undeca-1(EEZ) |
| 2-isopropyl-1-methoxy-4-methylbenzene | thymol methyl ether | 50.0 | 1209 | 1209 | | thymol methyl ether |
| thymol methyl ether | thymol methyl ether | 50.3 | 1224 | 1224 | 1222 | carvacrol methyl ether |
| β-funebrene | β-funebrene | 64.3 | 1411 | 1413 | 1411 | β-funebrene |
| widdrene | isobazzanene | 65.9 | 1430 | | 1430 | isobazzanene |
| | β-barbatene | 66.1 | 1433 | 1440 | 1440 | β-barbatene |
| trans b-farnesene | sesquisabinene B | 66.7 | 1456 | 1457 | | trans-b-farnesene sesquisabinene B |
| β-chamigrene | | | 1476 | 1476 | | β-chamigrene |
| α-farnesene | -farnesene | 69.0 | 1501 | 1505 | 1496 | α-farnesene |
| α-longipinene | | 69.6 | 1508 | | | α-longipinene |
| β-dihydroetarfuran | β-dihydroegarofur | 70.1 | 1500 | | 1500 | β-dihydroagurarofu |
| dill apiol | apiol | 75.6 | 1584 | | 1577 | dill apiol |

**Table 5:** Percent composition of *L. grayi* and *L. porteri*

| Compound (PBM) | % *L. porteri* | RI values *L. porteri* | % *L. grayi* | RI values *L. grayi* |
|---|---|---|---|---|
| α-thujene | 0.26 | 923 | 0.09 | 922 |
| α-pinene | 1.02 | 929 | 0.84 | 929 |
| sabinene | 0.03 | 940 | 2.03 | 941 |
| β-pinene | 15.56 | 972 | 0.35 | 972 |
| β-myrcene | 1.51 | 986 | 0.26 | 986 |
| α-phellandrene | | | 3.16 | 996 |
| α-terpinene | 0.09 | 1005 | 0.24 | 1008 |
| *p*-cymene | 5.2 | 1012 | 6.36 | 1012 |
| β-phellandrene | 0.29 | 1017 | 1.68 | 1019 |
| limonene | 0.4 | 1019 | 0.21 | 1021 |
| Z-β-ocimene | 0.63 | 1031 | | |
| E-β-ocimeine | 0.04 | 1041 | | |
| γ-terpinene | 2.68 | 1051 | 9.20 | 1057 |
| *p*-mentha-1,5,8-triene | 0.05 | 1063 | | |
| terpinolen/α-terpinolene | | | 1.21 | 1077 |
| α-thujone | 0.15 | 1081 | | |
| cis-thujone | 0.08 | 1091 | | |
| α-phellandrene epoxide | 0.23 | 1119 | | |
| viridine | | | 13.14 | 1132 |
| 4-terpineol | 0.06 | 1162 | | |
| thymol methyl ether | 0.06 | 1213 | 0.05 | 1209 |
| carvacrol methyl ether | 1.17 | 1225 | 0.46 | 1224 |
| cyclofenchene | 0.27 | 1250 | | |
| bornyl acetate | 0.2 | 1265 | | |
| sabinyl acetate | 23.18 | 1281 | | |
| β-funebrene | 0.07 | 1409 | | |
| issobazzanene | | | 0.10 | 1430 |
| β-barbatene | | | 0.13 | 1440 |
| β-selinene | 0.72 | 1480 | | |
| β-dihydroegarofuran | | | 0.22 | 1500 |
| dill apiole | | | 0.40 | 1584 |
| Z-ligustillide | 0.004 | 1682 | | |
| Unknown 1 | | | 3.82 | 1428 |
| Unknown 2 | | | 4.50 | 1675 |

### 3.3.3 Comparison of constituents from different *L. grayi* populations

The gas chromatogram pattern of the SAFE-BAENG extracts from five populations of *L. grayi* was compared qualitatively and quantitatively to determine if there is a difference between extracts based on either storage time before extraction or harvest location for each root sample (**Figure 26**). Population 1 was taken in a central location amongst groups of *L. grayi* plants at Gold Lake, while populations 2 through 5 were harvested about a quarter mile away from each other, heading south down the lake. The first *L. grayi* root population extraction was performed one year prior to all others, while roots from populations 2 through 5 were extracted (in numerical order) about 2-3 weeks after each other. Root samples produced almost identical chromatograms amongst populations, as shown in **Figures 22-24,** but did display a slight decline in intensity from population 1 to 5, as shown for certain peaks in **Figure 26**.

From these results, it is apparent that the extraction of the first population, which utilizes the freshest root sample of the five, yielded peaks slightly higher in intensity than the peaks of other populations. Either there were more volatile constituents in population 1 root or the absolute amount of volatiles is affected by the freshness of the root extracted. Also, since all chromatograms are very similar, there is no likelihood of contamination by other species along the bank of Gold Lake.

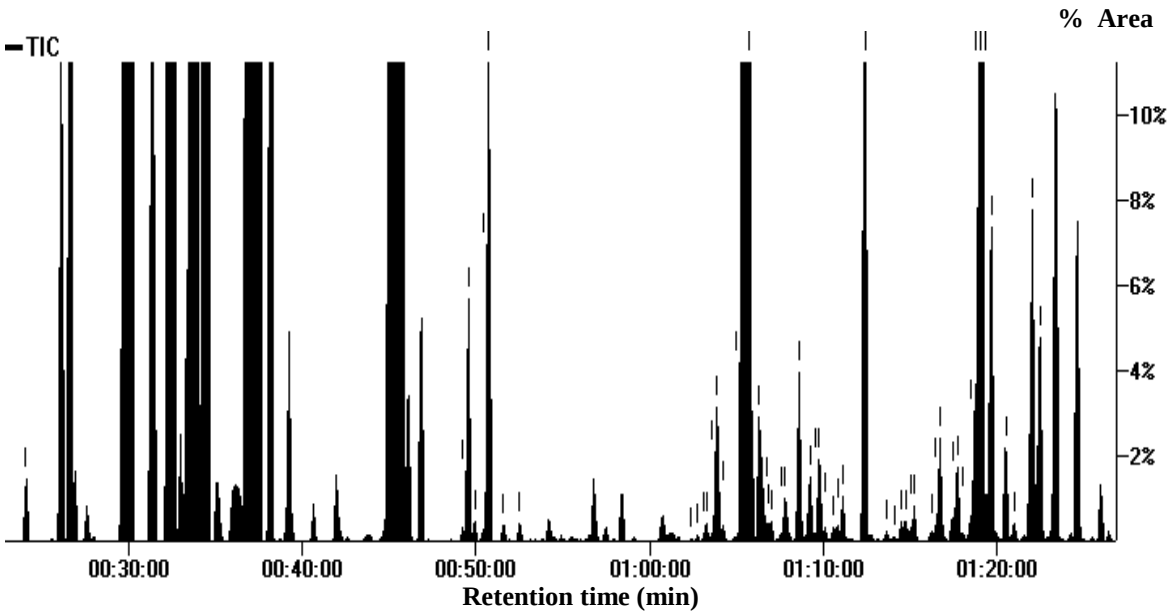


**Figure 22**: Gas chromatogram of SAFE extract of *L. grayi* population 2

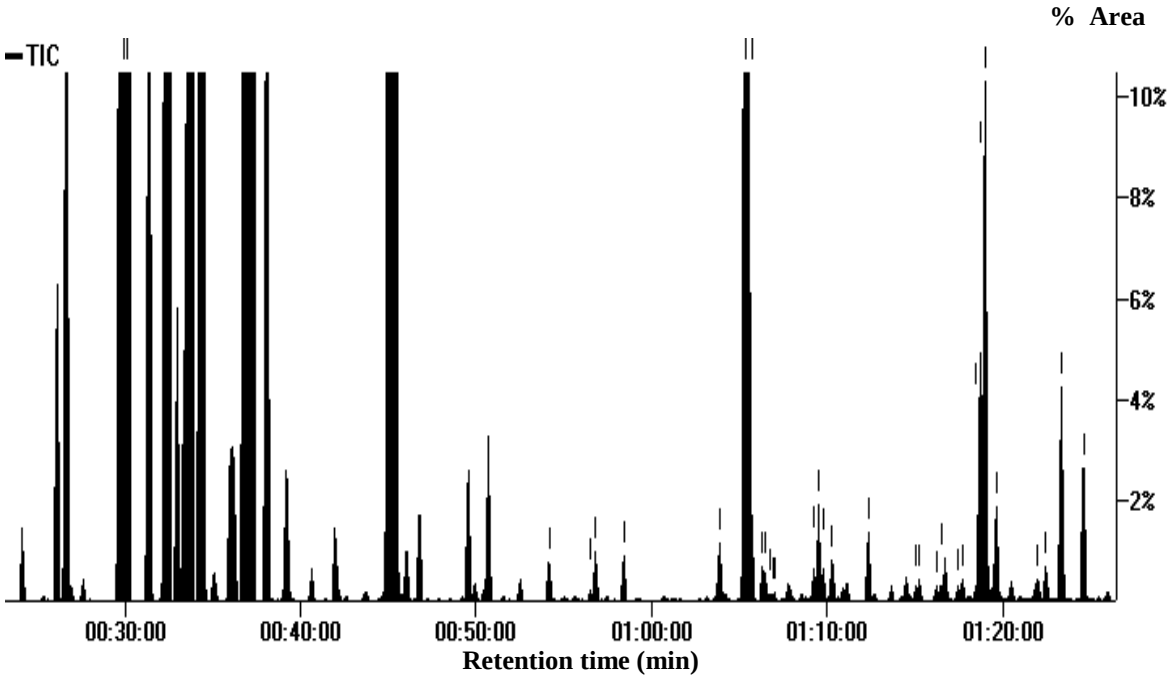


**Figure 23**: Gas chromatogram of SAFE extract of *L. grayi* population 3

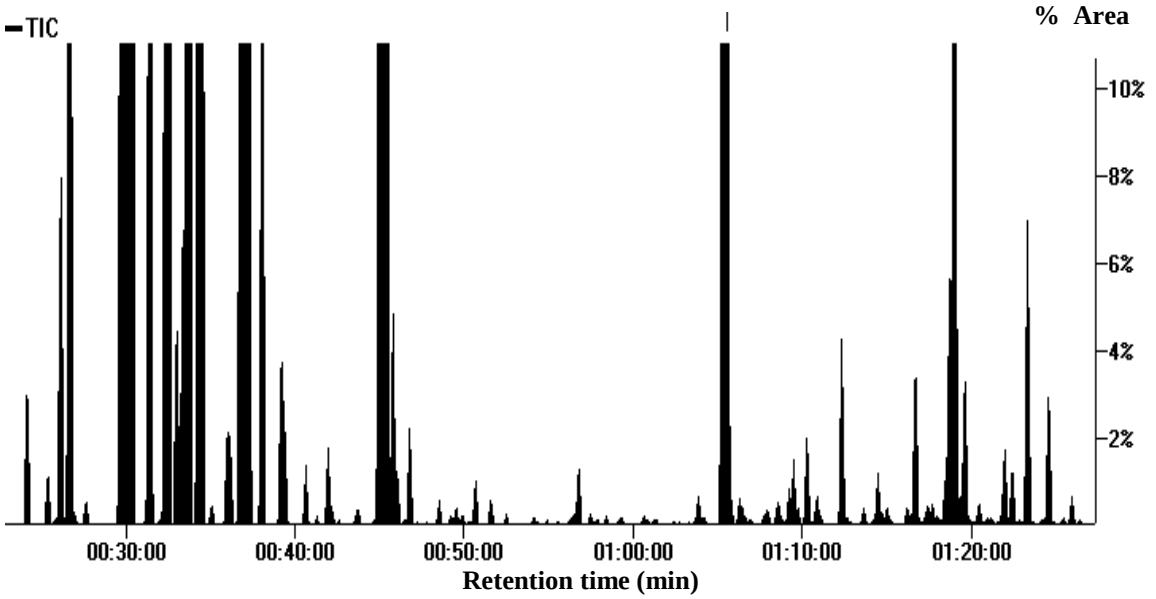


**Figure 24**: Gas chromatogram of SAFE extract of *L. grayi* population 4

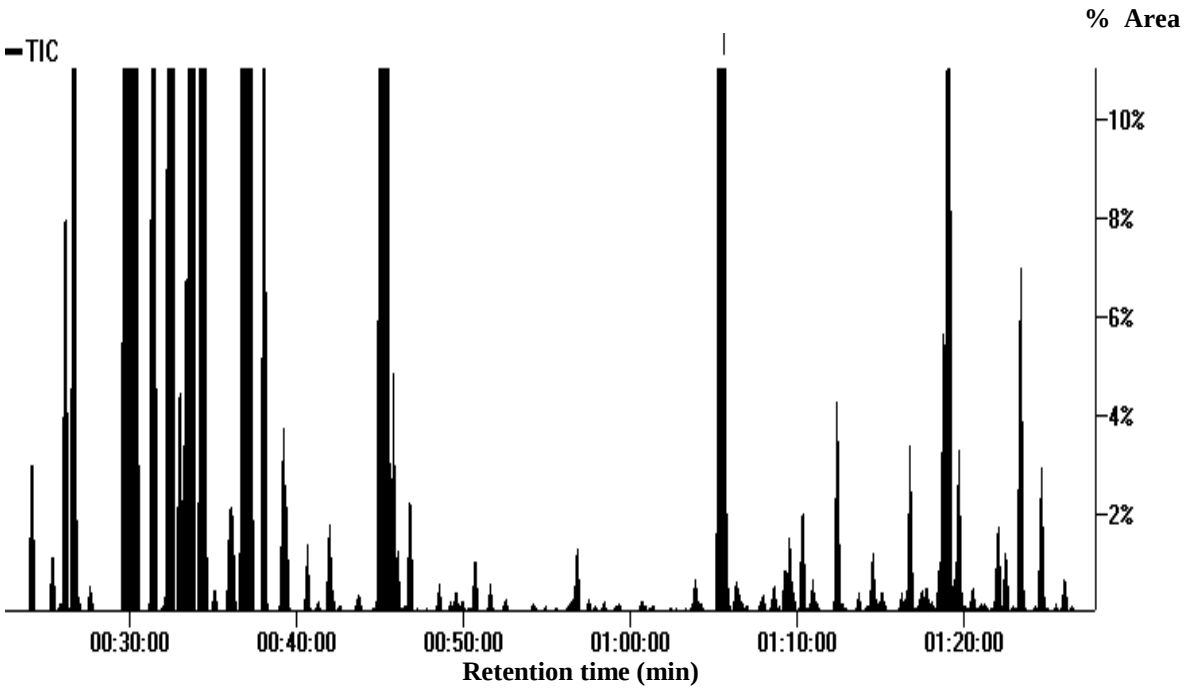


**Figure 25**: Gas chromatogram of SAFE extract of *L. grayi* population 5

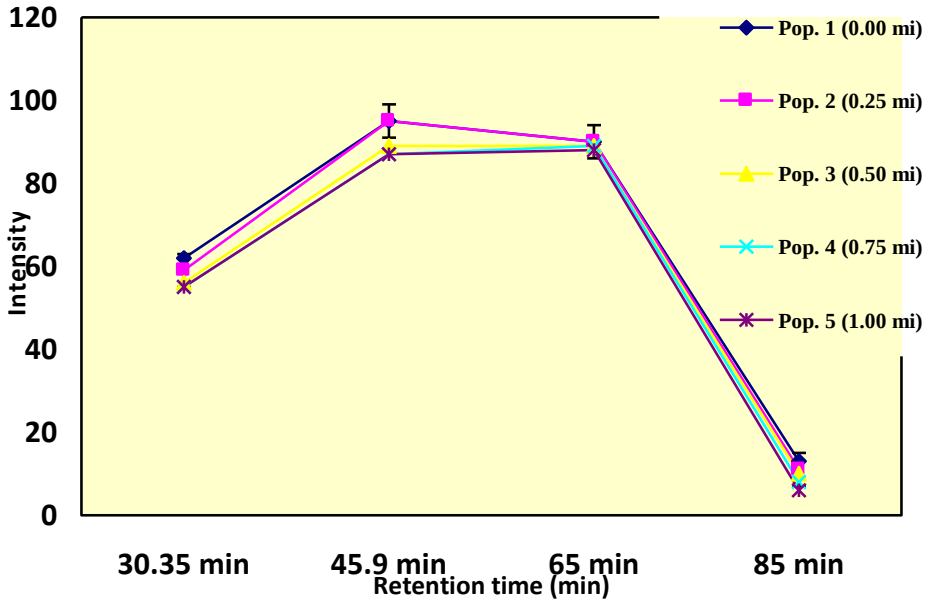


**Figure 26**: Peak area by % for β-pinene (30.35 min), viridine (45 min), and new unknown compounds 1 (65 min) and 2 (85 min)

# Chapter 4

# CONCLUSIONS

The constituents in *L. grayi* were with several extraction method: simple solvent extraction, soxhlet extraction, and SAFE-BAENG extraction, and many were identified through the comparison with *L. porteri*. Several large peaks representing unknown compounds were found and have undergone thorough identification by NMR in another study. Based on the findings from experiments, the following conclusions were drawn:

1. The effectiveness of the different solvents used for simple solvent extractions was hexane (8 peaks) > 95% ethanol (4 peaks) > dichloromethane (2 solvent peaks
2. The soxhlet extraction method is better than simple solvent extractions and is useful for extracting monoterpenes. The chromatograph produced 49 total peaks, with 12 peaks large enough to be tentatively identified in the *L. porteri* chromatogram, and 53 total peaks, with 16 tentatively identified peaks in the *L. grayi* chromatogram. Only 7 compounds identities were confirmed by retention time and MS comparison: α-thujene, α-pinene, sabinene, β-pinene, α-phellandrene, terpinene, and β-phellandrene.
3. Of all the extraction methods, SAFE extraction with the BAENG apparatus was the best extraction method, based on greater extraction of most volatiles in the compound, high sensitivity, less solvent waste, and most of all, characterization of larger hydrocarbons such as sesquiterpenes.

4. Disadvantages of SAFE-BAEG are the expensive, unique glassware, as well as having to carefully fill the compartments with liquid nitrogen and checking the machinery continuously for more than an hour during distillation. Furthermore, it is difficult to clean the condenser, so there can be a greater chance of environmental contamination.
5. The difference between the 5 populations of *L. grayi* extracted by SAFE-BAENG extraction shows that there is no likelihood of contamination along the bank of Gold Lake, but that volatility mat be affected by the freshness of root sample.
6. The SAFE-BAENG method produced 154 total peaks, 31 tentatively identified compounds, and 19 confirmed compounds for *L. grayi*; and 160 total peaks, 46 tentatively identified and 25 confirmed for *L. poteri*.
7. Most (95%) of the identified compounds in both plant roots are terpenes, and 64% of the constituents are the same, with 14 compounds in common. Of the shared compounds, most of them are monoterpenes, and a few are sesquiterpenes; thymol, and carvacrol methyl ether . The major compound in *L. porteri* is sabinyl acetate (23% of volatiles) and in *L. grayi*.is γ-terpinene (18%).
8. Several larger peaks representing unknown compounds were found and have undergone for further research through NMR for identification, resulting in a publication (Cool, et al, 2010) that proposes a new metabolic pathway.
9. *Z*-ligustilide, which has anti-cancer properties, is only present in *L. porteri* and was not found in *L. grayi*.

**4.1 Future work**

A better understanding of the differences amongst *L. grayi* populations can be further improved, with more time and resources, by considering other factors. First, the environment or climate can affect *L. grayi* root properties, so it would be interesting to examine *L. grayi* roots from Canada. Secondly, to better understand the ligusticum family's wide medicinal properties, it would ideal to compare the chemical constituents of *L. grayi* with many more ligusticum relatives besides that of *L. porteri*. Third, there is the issue of resolving the metabolic pathway(s) responsible for the irregular sesquiterpenoids.

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